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THE EXPERIMENTAL PRODUCTION OF EXOPHTHALMOS IN FUNDULUS BY MEANS OF ANTERIOR PITUITARY EXTRACTS[1]

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FOLLOWING the basic work of several investigators in establishing the relationship between the anterior pituitary and the thyroid, experimental hyperthyroidism was produced in many animals by implants, or by injections of suspensions and extracts of the hypophysis. Hyperthyroidism and concurrent exophthalmos, a situation resembling Graves' disease, was produced experimentally by injecting pituitary emulsions in ducks (Schockaert, 1931). Later, similar results were obtained in guinea pigs by Loeb and Freedman (1932), and Friedgood (1934).

The purpose of this paper is to report in detail a series of experiments wherein a remarkably rapid exophthalmos can be produced in Fundulus, the common Atlantic "minnow." Some of these experiments were done several years ago, and reported in abstract form (Abramowitz and Fevold, 1937; Abramowitz, 1940). In addition to these earlier experiments, recent observations[3] are also included.

MATERIALS AND METHODS

Healthy specimens of *Fundulus heteroclitus, Linn.*, measuring 3–4 inches in total length were used. The fish were kept in a stock tank of running tap

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water, the temperature of which averaged 10°C. in winter and 18°C. in summer. For individual experiments, the animals were maintained in aquaria at room temperature (20–25°C.).

All special methods will be dealt with under separate headings. However one general method—that of injecting the animals—will be described in detail here. Fundulus has a scaly integument over a rather firm body wall. Injections into the peritoneal cavity through the body wall always leave a small hole which remains open for considerable time following withdrawal of the needle. Because of this hiatus between the peritoneal cavity and the outside, a certain amount of the injected material is forced out through the hole during the swimming movements of the fish. In order to avoid such loss of fluid, which would defeat quantitative treatment, a method of intraperitoneal injection via the rectum was devised. The tip of the needle was in-

FIG. 1. Two exophthalmic fish (left and right) 12 hours after a single injection of sheep anterior pituitary extract. Normal fish in the center for comparison.

serted into the cloaca, and the syringe deflected so that it ran parallel to the long axis of the fish. The needle was then inserted through the rectum, over the pelvic girdle and into the peritoneal cavity for a distance of about one-half inch. Care was taken to keep the tip of the needle close to the abdominal wall, so that the viscera were not penetrated. After injection and withdrawal of the needle, the soft tissues of the rectum close completely and no loss of the injected fluid was ever encountered. About 10 animals can be injected per minute in this fashion. The volume of fluid administered varied from 0.1 cc. to 1.0 cc., the latter volume being maximal for the size of fish used. Usually, however, 0.5 cc. of fluid was injected.

EXPERIMENTAL

1. Production of Exophthalmos by Pituitary Extracts

Exophthalmos was first produced in Fundulus by impure preparations of follicle stimulating hormone (F.S.H.) and of luteinizing hormone (L.H.), made from whole pituitary glands of sheep. On the day following a single injection of either preparation at a dose of 1 gram equivalent (gm. eq.) of pituitary tissue, all fish became markedly exophthalmic (Fig. 1). Detailed observation showed that complete exophthalmos could occur in some fish as early as five hours following a single injection, but in most cases, the extent of the protrusion was maximal on the second or third day after a single injection. Exoph-

thalmos thus induced persisted for about two weeks after which the eyeballs reverted to their normal position. Furthermore, if another dose was given at this time, exophthalmos could again be induced.

2. Origin, Specificity and Tentative Identification of the Exophthalmic Factor.

An attempt to determine the specificity of the response and to identify tentatively the active principle was made by testing a variety of endocrine substances. These substances are shown in Table 1 where the results are reported in terms of whether exophthalmos was (+) or was not (−) produced. Dosages used varied from small to toxic except where the exact dose is given in the table.

TABLE 1

Substance	*Results*
Pyridine Extract of Sheep Pituitary Glands	+
Pituitrin (Parke, Davis & Co.)	−
Pressor principle (ibid.)	−
Oxytocin (ibid.)	−
Intermedin	−
F.S.H.	+
L.H.	+
Prolactin (60 I.U., Schering)	−
Follutein (P.U.) (300 I.U., E. R. Squibb & Son)	−
P.M.S. (10 I.U., Antex-Leo)	−
Growth Hormone (Parke, Davis & Co.)	−
Thyrotrophic Hormone (ibid.)	+
F.S.H. (from Urine of castrate women)	−
Methyl cyanide	−
Dessicated Thyroid	−
Thyroxine	−
Relaxin	−

From these results, it may be concluded that (1) the production of exophthalmos is a specific response to a constituent of pituitary tissue since proteins from other sources and certain pure proteins of the pituitary are ineffective; (2) the active principle arises from the anterior lobe; and (3) that the active principle is associated with the thyrotrophic hormone. This last conclusion is also based on the fact that all preparations (F.S.H., L.H., whole extract) which induced exophthalmos also produced hyperplasia and hypertrophy of the thyroid in hypophysectomized frogs and turtles. Those substances (P.U., P.M.S., F.S.H. from urine of castrate women) which did not produce exophthalmos, although given in doses equal in gonad stimulating ability to F.S.H. and L.H., had no thyroid stimulating ability.

3. Assay of the Exophthalmic Factor.

One hundred forty animals were divided equally among 7 tanks. Various dosages of a pituitary extract (extract number 22p, to be described later) from 200 mg.eq. of fresh pituitary tissue to 1 mg.eq. per group were injected in a volume of 0.5 cc. The number of exophthalmic fish at various time intervals following the injection was noted and recorded as percentage positive response. With these doses

various grades of exophthalmos ranging from minimal to complete, protrusion were encountered. For many reasons it was decided to include as positive all fish which showed a definitely perceptible exophthalmos.

The results of this series are shown in Figure 2, where the percentage of positive response is plotted at various time intervals following a single injection of different amounts of pituitary extract. From this curve, it can be seen that the reaction as measured by the num-

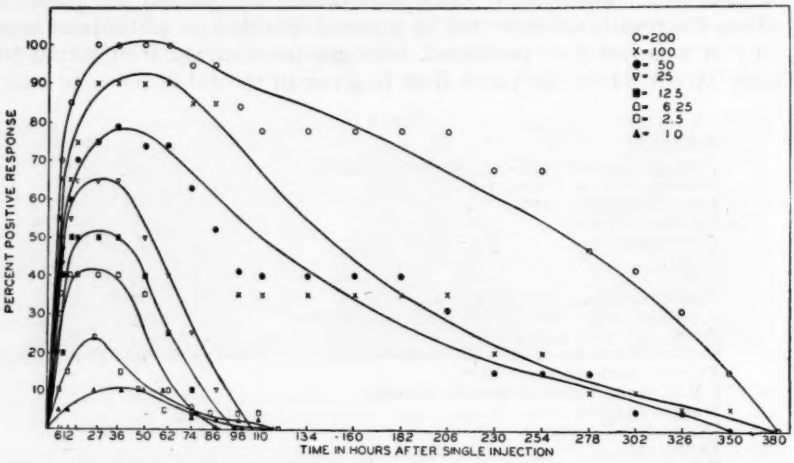

FIG. 2. The percentage of exophthalmic fish at various time intervals following a single injection of different amounts of sheep pituitary extract (22p). Numbers in top right indicate dosages in milligrams equivalent. (Each point represents 20 fish.) Temperature in aquarium was 23–25°C.

bers of animals becoming exophthalmic is greatest at approximately one day. The rate of response, however, is greatest during the first 10 hours after the injection. The time at which all the eyeballs revert to their normal position varies considerably, but is roughly proportional to the dosage employed.

A similar experiment in which readings were obtained at 3, 6, 9, 12 and 24 hours following injection was done to ascertain more definitely the speed at which the reaction occurs, and to choose a suitable time at which the readings should be made. The results are shown in Figure 3, where it can be seen that the rate of the reaction for higher doses is maximal and constant towards 3 hours, and for small doses around 9 hours. However, 6 hours is a time at which the rates are maximal and constant for most dosages, and it was decided to take all readings at this time. One further experiment was done to determine whether the induced exophthalmos was constant or vacillated from time to time. A group of 20 animals was injected with a dosage of 12.5 mg.eq. (see Fig. 2), which induces exophthalmos in

about half of the fish. The animals of this group were observed individually at 3, 6, 9, 12 and 24 hours for the 1st day, and thereafter, every twelve hours for 5 days. Records were kept of the severity of the exophthalmos by grading the reaction from mild to complete in numbers of from 1 to 5. The results showed that fish which did not respond within the first day remained normal for the entire period. Animals that became exophthalmic remained so for several days. At

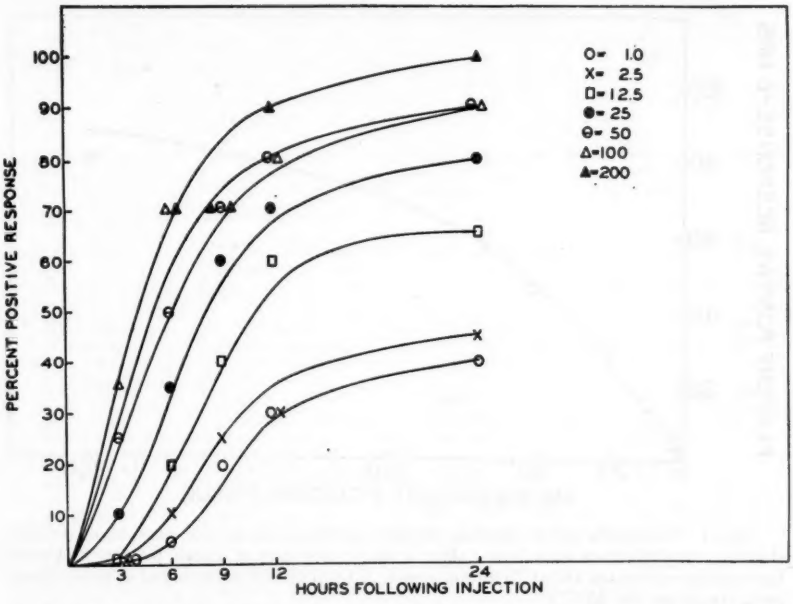

FIG. 3. Percentage of positive response (fish showing exophthalmos) during the first twelve hours following a single injection of sheep pituitary extract (22p.) Dosages are in mg.eq. Each point represents 10 animals. Temperature in aquarium 19°C.

no time did an exophthalmic animal become normal and then proptotic later, indicating that the eyeballs do not oscillate between the normal and the proptotic position during the reaction. After the first day the severity of exophthalmos in many animals may become even greater, but no further increase in the number of exophthalmic fish occurs.

The effect of dosage on the percentage of animals showing exophthalmos at 6 hours following a single injection is shown in Figure 4. This curve is a composite of all results involving quantitative determinations (430 animals) obtained under roughly equal conditions (Temp. 19–25°C.). On the basis of this curve a unit of exophthalmic activity was defined as that amount of substance causing exophthalmos in about 25% of the animals 6 hours following a single intra-

peritoneal injection. This point was chosen because it lies in the region where change in response per unit change in dosage is maximal. Moreover, a qualitative change in the response of the eyes occurs at this dosage level. The amount of substance that causes proptosis in 25% of the fish induces a minimal exophthalmos at 6 hours. A dosage twice this unit amount affects almost twice as many fish (around 47%) but also induces a more severe exophthalmos in some of the animals.

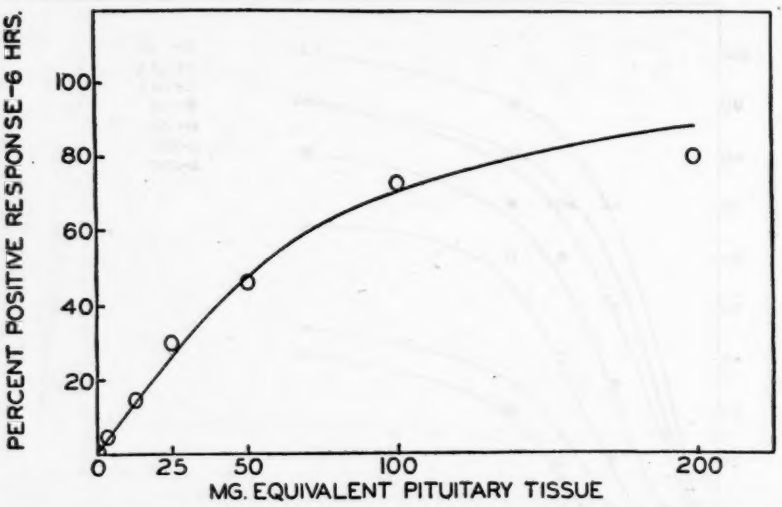

FIG. 4. Composite curve showing relation between dosage and percent of animals showing exophthalmos at 6 hours after a single injection of sheep pituitary extract. Each point represents about 70 animals each, a total of 430 animals being used. (Temperature range 19°–25°C.)

Doses half the amount needed for a 25% response affect less than half as many fish (10% or less) and the proptosis induced is minimal. Hence, by noting the severity of the reaction in addition to determining the percentage of animals showing a response, an additional check on the assay is obtained. Routine assays were performed by determining on a small number of fish, usually 8, the amount of substance producing a 25% response. The test was then repeated with three groups of twenty animals each, the groups receiving respectively doses equal to, one-half, and twice the roughly determined unit dose. The responses induced by these three dosages were compared with the smooth curve (Fig. 4) and the number of units calculated. Activity will be expressed as exophthalmic factor units (E.F.U.), as described above. The accuracy, and the sensitivity (i.e., the ability to discriminate between small differences in dosage) of this method has not been studied.

4. Fractionation of Pituitary Extracts.

A systematic fractionation of six hormones of the anterior pituitary glands of sheep has been published by Fevold, Lee, Hisaw and Cohn (1941). The method of fractionation consisted essentially of preparing an extract at pH 8.0, which was then separated at pH

TABLE 2

Fractions	Hormones of the anterior lobe	E.F.U./kg. pituitary tissue prep. #1	prep. #2
pH 8.0 Extract	Growth, Adrenotrophin, Prolactin, L.H., F.S.H., Thyrotrophic	25,000	10,000
pH 8.0 Residue	Not tested for above	Neg. @ 1.0 gm. eq.	Neg. @ 1.0 gm. eq.
pH 5.4 solution in M/4 $(NH_4)_2SO_4$	Growth, L.H., Thyrotrophic, F.S.H.	20,000	10,000
pH 5.4 Ppt.	Adrenotrophin, Prolactin	Neg. @ 1.0 gm. eq.	Neg. @ 0.4 gm. eq.
1.2 Molar Ppt.			
H_2O sol.	traces L.H.	Neg. @ 1.0 gm. eq.	Neg. @ 2.5 gm. eq.
H_2O insol.	growth hormone	Neg. @ 1.0 gm. eq.	Neg. @ 2.5 gm. eq
1.6 Molar Ppt.			
H_2O sol.	L.H. (25%)	1,000	Neg. @ 2.5 gm. eq.
H_2O insol.	Growth hormone	Neg. @ 1.0 gm. eq.	Neg. @ 2.5 gm. eq.
2.0 Molar Ppt.			
H_2O sol.	L.H. (65%), Thyrotrophic (30%)	2,500	2,600
H_2O insol.	none	Neg. @ 4.0 gm. eq.	Neg. @ 2.5 gm. eq.
2.4 Molar Ppt.			
H_2O sol.	L.H. (10%), Thyrotrophic (70%) F.S.H. (traces)	15,000	5,000
H_2O insol.	none	Neg. @ 4.0 gm. eq.	Neg. @ 2.5 gm. eq.
2.8 Molar Ppt.			
H_2O sol.	F.S.H.	1,000	500
H_2O insol.	none	Neg. @ 4.0 gm. eq.	Neg. @ 2.5 gm. eq.
3.8 Molar Ppt.			
H_2O sol.	none	Neg. @ 1.0 gm. eq.	not tested

5.0–5.4 in M/4 ammonium sulfate into isosoluble and isoelectric portions. The pH 5.0–5.4 solution was then fractionated by dialyzing various amounts of ammonium sulfate into the solution and removing precipitates thus formed. Each precipitate was then dialyzed against water so that water soluble and water insoluble portions were obtained. All fractions were then injected into the fish at various dosage levels until unit doses were found. When large doses of certain fractions, as measured in gram equivalents of original pituitary tissue were negative for E.F., they were increased until toxic to the ani-

mals.[4] Table 2 is a summary showing the distribution of the six anterior lobe hormones, taken from the data of Fevold, Lee, Hisaw and Cohn (1940), and the distribution of E.F. as determined by the method of assay described in the previous section.

From the results listed in Table 2, it can be seen that about one-third of the exophthalmic activity was lost during fractionation. In the main, however, there was general agreement in the activity of the various fractions in both experiments as far as exophthalmogenesis was concerned. A significant finding was that none of the globulin portions of the various salt fractions was active at doses one hundred times greater, in some instances, than the unit dose for producing exophthalmos;—emphasizing again the specificity of the response. The major finding was that the distribution of E.F. throughout the procedure was parallel to only that of the thyrotrophic hormone. This does not necessarily imply any identity between the two hormones since the water soluble component precipitated between 2.0 and 2.4 M. $(NH_4)_2SO_4$ is a mixture of many proteins some inert and some possibly active principles for which no tests were made.

A rapid method of preparing E.F. was devised on the basis that most of the active principle was precipitated between 1.5 and 2.5 M. $(NH_4)_2SO_4$. The pH 5.4 solution was brought immediately to 1.5 M, and the precipitate rejected. The salt concentration of the solution was raised to 2.5 M, and the precipitate dialyzed until salt-free. Insoluble material was removed by centrifuging. The resulting solution was clear, fairly stable and contained 2 gm.eq. per cc., or about 40 E.F.U. per cc. Eight such preparations (number 22p) have been made with activities ranging from 10 to 80 E.F.U. per cc. of solution.

5. Relation of the Thyroid to Exophthalmos.

Although it was shown that administered thyroid hormone could not induce the exophthalmic reaction, it was of interest to determine if any morphological changes occurred in the thyroids during experimental exophthalmos. The thyroid in Fundulus is scattered along the floor of the pharynx which forms a connecting cartilaginous bar for the ventral attachment of the gill arches. Many of the follicles lie close to and intimately surround the great vessels supplying the gills.[5] Serial sections show that the follicles are located mainly between arch 2 and 5.

The relation of the thyroid to experimental exophthalmos was studied by giving a single injection of 200 mg.eq. of 22p to a group of 30 fish. Two fish were killed at 3, 6, 9, 12, 24 hours for the first day, and thence daily for 7 days following the injection. All fish showed exophthalmos of varying grades at these times. The floor of

[4] Doses in Table 2 noted as negative represent largest doses tolerated by the fish.
[5] Thus thyroidectomy in this fish seems impossible.

the pharynx was sectioned serially between arch 2 and arch 5 at 10μ thickness, and stained with Delafield's hematoxylin and eosin.

The histological structure of the thyroid in Fundulus is somewhat variable but as a rule consists of fairly large acini with uniformly staining colloid, in most cases, sharply abutting the follicular wall. The follicle is composed of cuboidal epithelium whose cytoplasm stains in a slightly acidophilic manner. The luminal edge of the epithelium is usually sharp. The epithelial cells may be no taller than the length of their nuclei while in others, the nuclear length may be about 75–80% that of the height of the cell. The nuclei are irregularly round to oval in shape, and contain darkly staining chromatin tending to be disposed along the periphery, close to the nuclear membrane. There is usually no visible nucleolus. On the whole, the normal thyroid is relatively avascular.

In the experimental animals, no change in histological structure of the thyroid could be discerned three hours following injection, although exophthalmos was decidedly present. There occurs, however, an increased vascularity of the gland. Six hours following injection there appears to be a further increase in vascularity of the gland and a few minor changes in the appearance of the follicles are evident. The acini tend to be slightly irregular in shape. The cytoplasm of the follicular cells is less acidophilic in staining reaction, and the nuclei look vesicular and more lightly stained than normal. At thirteen hours following injection, the nuclei show a definite nucleolus.

Unquestionable alterations in the histological appearance of the thyroid can be noted about 24 hours following injection. There is less colloid, which is faintly stained and considerably vacuolated. Vascularity has increased to a maximal extent. The follicular cells are taller, and the cell height may be twice that of the nucleus. In some areas the luminal edge of the cell is frayed. The nucleus is in a basal position, and is turgid. The chromatin, which in unstimulated cells is coarse, now assumes a fine, reticular state and is situated close to the nuclear membrane. The gland as a whole seems adenomatous

Two or three days following injection, there is a marked hypertrophy and hyperplasia of the thyroid. The acini are distorted and all the colloid has disappeared. The cell height may be four to five times the nuclear length. In some sections vacuoles containing colloid appear to be discharged from the cell into the lumen.

From this account, it can be concluded that exophthalmos may precede by several hours any obvious change in the histological appearance of the thyroid. Although there seem to be some minor changes in the gland, those which are usually regarded as indicative of thyroid stimulation do not appear until about 12 hours following a single injection. Consequently, it would appear, at least superficially, that proptosis does not depend on activation of the thyroid.

6. Mechanism of Exophthalmos.

A. Histological Alterations in the Orbital Tissues.

Serial sections of the heads of normal and exophthalmic fish showed several marked differences in the orbital tissues. The most obvious change in the exophthalmic fish was the protrusion of the conjunctiva along its entire circumference beyond the orbital wall, a situation which never occurs in normal animals. The eyeball itself protrudes about one-half centimeter beyond its normal position. The conjunctiva appears much swollen and oedematous in the experimental animals, and consists of loose areolar tissue strands separated by large clear spaces. The extra-ocular muscle fibers do not appear cytologically different from normal. However, the muscle bundles of the experimental animals are more widely separated from each other because of an oedematous interstitium. The entire muscle also seem stretched and taut. Within the eyeball there are hardly any differences save that the inner areolar layer of the sclera appears more watery than in normal eyeballs.

The most striking changes are within the orbital tissues. In exophthalmic fish, there is a large triangular-shaped mass, containing a few leucocytes and lymphocytes. This mass which seems to represent a fluid precipitated by the fixing agent abuts against the eyeball with the base of the triangle against the sclera, and with its apex bordering that of the orbit. The impression obtained is that this mass is actually pushing the eyeball out of the orbital cavity. Only a small amount of this material is present in the normal fish. The orbital fat and areolar tissue in the experimental animals is much swollen and oedematous, and infiltrated by lymphocytes.

B. Presence of Free Retrobulbar Fluid within the Orbit.

The suggestion from histological study that a fluid was present in the orbits of exophthalmic animals led to a macroscopic search of the orbit. If the conjunctiva of a normal fish is punctured, a drop of clear fluid may escape. If the same procedure is carried out in an exophthalmic fish, a greater amount of fluid escapes with a rush as if a cyst had been pricked. Thus, in exophthalmos, considerable pressure must be present within the orbit. With a fine needle, 0.01 cc. of fluid could be aspirated from normal orbits but the orbits of exophthalmic fish readily yielded about 0.05 cc. of a clear, glassy and somewhat sticky fluid, which superficially resembled lymph.

Immediately the orbit is aspirated or the fluid drained after opening the orbital cavity, the proptosis completely disappears. This regression of the eyes is so prompt that the escape of fluid and recovery from exophthalmos are coincident phenomena. If the aspirated fluid or a similar amount of distilled water is then reinjected into the orbit, the eyeball is immediately thrust into its exophthalmic position. It thus appears again that the retrobulbar space is under high pressure which if released allows the eyeballs to fall, or be pulled, back into their normal position by the taut extra-ocular muscles.

Thus, the mechanism of exophthalmos in Fundulus can be visualized as being basically one of increased intraorbital pressure. The pressure is exerted by a fluid secreted into the retrobulbar space presumably by the orbital tissues. Since the globe is enclosed on almost all surfaces by the bony orbit the pressure would be exerted behind the globe, thrusting it forward out of the orbit when the pressure had overcome the resistance of the extraocular muscles. Since the exophthalmos recedes, as shown by the curves of Figure 2, it must follow that the fluid is gradually absorbed, thus causing a fall in retrobulbar pressure and thereby allowing the eyeball to return to its normal position. This hypothesis was put to test in several ways.

It was reasoned that if proptosis was due to the formation of fluid, and the fluid was not allowed to accumulate, proptosis should be prevented. Consequently, the right orbit of a group of normal fish was decompressed by slitting the conjunctiva along the dorsal half of the orbital circumference so that a semicircular opening was present between the orbital cavity and the outside. None of the superior orbital muscles was injured. The fish were then given 200 mg.eq. of extract 22p. In this manner, the left or unaltered eye may be made exophthalmic whereas the decompressed right eye remained in its normal position. The same results may be obtained if a small drain is inserted in the retrobulbar space of the right eye, leaving the left eye as a control. The failure of exophthalmos to occur in decompressed orbits would indicate that a fluid material had escaped through the artificial opening and no longer could exert pressure upon the eyeball.

The corollary of the hypothesis demands that the fluid once formed be absorbed to account for the regression of the proptosis. To show that orbital tissues do absorb fluid, 0.1 cc. of various fluids was instilled into the retrobulbar space of the right eye. The left eye was watched as a control, and observations made daily for twelve days. Both eyes were graded for exophthalmos from 1+ (minimal) to 5+ (maximal), and records kept of each eye in individual fish. Four groups of animals, with ten fish per group, were studied. Group 1 received 0.1 cc. Ringer's solution in the retrobulbar space of the right eye; Group 2, 0.1 cc. pituitary extract (22p); Group 3, 0.1 cc. of retrobulbar fluid aspirated from the orbits of exophthalmic fish; and Group 4, 0.1 cc. sesame oil. As was stated previously, instillation of fluid induces an immediate maximal, artificial exophthalmos.

In Group 1 (Ringer's solution) the artificial exophthalmos begins to regress within a few hours. Within 18 hours it had almost completely disappeared. Within 24 hours, the proptosis was completely gone. Although the animals were observed for twelve days, there were no further changes. The left eye showed no change at the moment of induction of the mechanical proptosis of the right eye or in any phase during and after the regression.

Group 2, receiving pituitary extract, showed rather interesting results. The artificial exophthalmos produced instantly on injection

began to regress, much like the group injected with Ringer's solution. Within about 10 hours, however, a recrudescence in exophthalmos occurred, so that the eyes became more proptotic during the next 2 days than they were at 10 hours after injection. Over the remaining 10 days, the proptosis gradually declined so that the right eyes of the entire group became normal some twelve days after instillation. The interesting finding was that 18 hours after instillation of the right orbit, the left eye became proptotic, however, in lesser degree. From this point on, the proptosis of the left eye followed the same course as that described for the right eye with the exception that the average daily measurement showed the proptosis of the left eye to be about one degree less than the right, and that all left eyes became normal about 10 days after the beginning of the experiment as compared with 12 days for the right eye.

Group 3 received instillations of the retrobulbar fluid aspirated from orbits of several hundred exophthalmic fish. The artificial proptosis of the right eye, like that of the preceding two groups shortly began to recede. Eighteen hours after instillation, the right eye of this group had fallen from an average 5+ artificial exophthalmos to 1.5+, precisely the same as saline. However, at 25 hours following injection, the right eyes became more proptotic (2.5+). This resurgence of exophthalmos was like that in the fish receiving pituitary extract, but it was less severe and lasted only 3 days, after which the right eyes became normal. The left eye in this group also became proptotic about 25 hours after the beginning of the experiment, attained an average 1+ proptosis which after one day, completely disappeared.

These results were interpreted as follows: The orbital tissues handled retrobulbar fluid artificially instilled in much the same way as saline for the first 18 hours, but after this time, the retrobulbar fluid behaved like pituitary extract in causing an increase in exophthalmos. Furthermore, like pituitary extract, the left eyes also became exophthalmic 6–12 hours after the recrudescence occurred, the only difference being that the severity and duration of the exophthalmos in both eyes of retrobulbar fluid treated animals were less than that of the corresponding eyes of fish receiving pituitary extract. This obviously meant that retrobulbar fluid siphoned from exophthalmic fish contained some of the activity of the pituitary extract with which the fish were injected intraperitoneally. Consequently, a routine six-hour assay was performed on the siphoned fluid in the usual manner, and it was found that there were 4 E.F.U. per cc. fluid. The difference in severity of all phenomena described between Group 2 and Group 3 can be attributed to the fact that Group 2 received 4 E.F.U., in the right orbit, whereas Group 3 received 0.4 E.F.U., present in the instilled retrobulbar fluid.

Group 4 received 0.1 cc. sesame oil into the right retroglobal space, causing the usual mechanical proptosis. Because this type of fluid is

poorly if at all absorbable in the fish, the mechanical 5+ proptosis remained without any signs of amelioration for as long as the animals lived. The left eye remained entirely normal.

All of these observations are understandable if it is assumed that the position of the eyeballs is the reflection of varying grades of intra-orbital pressure. Fluids placed in the retroglobal spaces cause instantaneously a maximal proptosis due to pressure artificially created. Fluids like saline, pituitary extract, and the retrobulbar fluid of exophthalmic orbits (presumably lymph) are absorbable, and as they are being absorbed the pressure decreases and the proptosis diminished. Saline is completely absorbed, and the exophthalmos disappears. Pituitary extract, presumably, acts directly on the orbital tissues, hence they form their natural fluid, increasing pressure, and causing an exacerbation of proptosis. Some pituitary extract has, however, been absorbed into the general circulation; hence after a slight delay, the left or opposite eye also becomes proptotic. The action of retrobulbar fluid aspirated from exophthalmic fish is qualitatively but not quantitatively the same as pituitary extract, a situation which is understandable on the assumption that the fluid contains some of the hormone injected intraperitoneally and distributed freely among body fluids. Finally, oil causes a permanent artificial exophthalmos when applied locally because it cannot be absorbed and thus maintains a permanent retroglobal pressure.

C. Miscellaneous Factors Affecting Exophthalmos.

Since exophthalmos in mammals has been reported following stimulation of the cervical sympathetic nerves and is said also to disappear under anesthesia and after death, a few experiments dealing with these factors were performed in Fundulus.

The right anterior sympathetic chain was sectioned in five animals. This procedure denervates the entire anterior right quadrant of the animal as far as any sympathetic activity is concerned. In animals prepared in this fashion, 200 mg.eq. doses of extract 22p produced the usual bilaterally equal exophthalmos. Five animals rendered proptotic were completely anesthetized with ether without in any way influencing the exophthalmos. Measurements of the exophthalmos by the skull-limbus technique showed no changes during and recovery from anesthesia. Five exophthalmic fish were killed by cutting out their hearts, and others were killed by destruction of the spinal cords. No changes in exophthalmos could be observed, even several hours after death. Whatever influence these miscellaneous procedures have on mammalian proptosis, their effect in Fundulus is lacking or insignificant.

DISCUSSION

Exophthalmos in fish is not unknown. An endemic disease consisting of thyroid hyperplasia, exophthalmos, muscular weakness and

emaciation, was reported by Hamre and Nichols (1926) in the fry and adult trout of a Wisconsin State Fish Hatchery. This condition, which has some obvious counterparts of Graves' disease, was cured by the administration of iodine to the hatchery water supply. Other reports of proptotic fish can be found in the literature but these are often unilateral and are due to infection, injury, parasites or neoplasia of the orbital tissues. It has been noted that goldfish undergo a metamorphosis wherein changes in body pigmentation and proptosis occur. Fish with telescopic eyes are well known, but these are permanent, and in many cases, adaptive affairs and are due to anatomical and genetic characteristics. However as far as the author is aware, experimental production of exophthalmos in fish by means of pituitary extract has not been previously reported.

Some effort has been expended in an attempt to decide whether E.F. can be identified with any known pituitary hormone. It seems to be a general belief that the thyrotrophic hormone causes exophthalmos by some direct action on the orbit, but examination of most data purporting to support this belief indicates that the evidence is mainly circumstantial. The preparations used in the past to induce proptosis consist of precipitates of crude extracts or whole pituitary emulsions, and it is hardly logical to conclude that although such preparations contain thyrotrophic hormone the resulting exophthalmos was due to contained thyrotrophic principle. It is also often assumed that thyrotrophic hormone produces exophthalmos because of the similarity between exophthalmic goitre and experimental exophthalmic hyperthyroidism. The idea that the action on the eyes is direct is deduced from the production of proptosis in thyroidectomized animals (Marine & Rosen, 1934; Smelser, 1937, 1939) and the occurrence of exophthalmos in humans without hyperthyroidism, in hypothyroid states, and after subtotal thyroidectomy (Means, 1944). Assays for endogenous thyrotrophin in the urine and blood of patients with various pituitary and thyroid disorders may also be construed as favoring the idea that thyrotrophic hormone produces exophthalmos. While such a belief may be entirely correct it must be emphasized that since thyrotrophic hormone of sufficient chemical purity has not been prepared and tested for exophthalmogenic properties, the available evidence is equivocal. The criticism of deductions based on bioassays for thyrotrophin in various states of disease would imply that if the pituitary could vary its secretion of this hormone, it could vary a hypothetical and distinct substance, E.F. The data in this paper, as well as Aird's (1940) work, show that the substance which induces exophthalmos (E.F.) and the thyrotrophic hormone are located in the same mixture of pituitary proteins. Further attacks on this mixture are necessary to ascertain whether these substances are or are not identical.

In comparison with other animals, the exophthalmos of Fundulus

can be produced within three hours and with minute amounts of pituitary tissue (10 mg.eq. of a crude extract or less) whereas in warm-blooded animals, chronic treatment for 3–8 weeks and amounts of pituitary emulsion as high as 15,000 mg. and preparations of tremendous doses have been used. The amount of sheep pituitary material usually needed to elicit one unit of exophthalmic activity in Fundulus contains about 0.1 chick unit (Bergman and Turner, 1939) of thyrotrophin. Since the weight of a fish is about one-fifth that of the chick, the Fundulus test on a weight basis is roughly the same order of sensitivity (i.e., the smallest amount that can be detected) as the chick and the guinea pig assuming for the moment that the Fundulus reaction is a test for thyrotrophic hormone. However, the total amount needed for an assay in Fundulus is about one-tenth that needed for the chick test. Thus, the method described would facilitate the detection of minute amounts of hormone found in urine, blood and other tissues in normal and pathological states.

Concerning the mechanism of the exophthalmos little more can be added. The pituitary is not needed for the response since exophthalmos may be induced readily in hypophysectomized Fundulus. Ablation of the cervical sympathetic makes no difference as far as the production of proptosis is concerned. The thyroid is probably not involved for the smallest doses which produce exophthalmos cause no simultaneous morphological changes in the thyroids, and thyroid therapy by itself does not produce the reaction. The gonad does not seem to be involved because active preparations of E.F. have been made with little or no gonadotrophic material, and because proptosis can be induced in mature and immature male and female specimens much before any conceivable gonadal stimulation can occur. The participation of glands such as the adrenal and thymus has not yet been investigated.

It is therefore believed that in Fundulus at least the pituitary principle acts directly on the orbital tissues causing oedema of the areolar and fat tissue, and the formation of free retrobulbar fluid. It has been shown that the fluid formed is under considerable pressure which pushes the eyeball forward because, being enclosed on all other sides by a firm body wall and by the bony orbit, this is the only way it can move. The remission of exophthalmos is due to absorption of the fluid.

The relationship of experimental exophthalmos in Fundulus to human exophthalmos is problematical since there is still much uncertainty with regard to the etiology and pathology of human proptosis, especially, as it occurs in Graves' disease. For a discussion of the latter topic the series of papers by Mulvany (1944) may be consulted. There is one aspect of human exophthalmos, however, that is not often encountered in experimental work, namely the existence of unilateral or unequal proptosis. In an earlier report, it was stated that exoph-

thalmos in Fundulus was always equal, complete, and bilateral. The earlier dosages were excessive, and all grades of exophthalmos from mild to severe have been since induced by appropriately lower amounts of E.F. But whatever the severity of the protrusion, the exophthalmos is always bilaterally equal during the development of the reaction. During recession, however, a slight inequality of the eyes may occasionally be seen. If the mechanism of exophthalmos has any counterpart in mammals, it is not difficult to understand why an unequal or a unilateral proptosis may develop, since unequal amounts of fluid may be formed, or unequal amounts of retrobulbar fluid-forming tissue may be present. During recession, fluid may be resorbed faster in one orbit than the other. Any or all of these possibilities could account for unequal proptosis.

The histological alterations in the orbital tissues of proptotic fish are quite similar to those reported for mammals, and to most reports dealing with varieties of human exophthalmos. There is one chief difference, however. To my knowledge the presence of free retrobulbar fluid has not been described in mammalian or human proptosis. If present, it must be an early phenomenon, and may have escaped detection. What the exact nature of this fluid in Fundulus is, where it is formed, and what its ultimate fate is are at the moment unknown.

SUMMARY

Bilateral and equal exophthalmos can be produced in Fundulus within a few hours after the injection of small amounts of sheep pituitary extract. The reaction occurs within 3 hours of injection and rapidly increases in both severity and numbers of animals affected during the first day, after which the exophthalmos recedes. When the eyeballs revert to their normal position, they may be made proptotic again by another injection of pituitary extract.

The time at which exophthalmos appears, the speed and severity of the reaction, the time at which the rate of response becomes maximal and constant, and the duration of the reaction are all proportional to the dosage of extract given. Curves relating these aspects of the experimental proptosis with time and dosage are presented. A method of assay of the pituitary substance inducing exophthalmos has been worked out. A unit of exophthalmogenic activity has been defined of that amount of substance which induces proptosis in one quarter as a group of 20 fish 6 hours following injection.

The exophthalmos is not produced by any known hormone of the posterior or intermediate lobe, and therefore must be due to a principle derived from the anterior lobe. Sheep pituitary tissue was therefore fractionated and six recognized hormones separated. The exophthalmic factor paralleled only the thyrotrophic fraction qualitatively and quantitatively. Many other fractions so derived are ineffective in inducing proptosis even though doses one hundred times greater

than the effective unit dose of the unfractionated extract are given. The reaction is therefore due to a specific constituent of the pituitary, and is either the thyroid stimulating hormone, or a new substance, showing properties thus far similar. A rapid method of preparing the exophthalmic factor is described.

The experimental exophthalmos in Fundulus is probably not dependent on the thyroid since the reaction occurs many hours before visible evidence of thyroid activation is obtained. Thyroid and thyroxine are ineffective in inducing proptosis. The hypophysis is likewise unessential, as is the presence of a functional sympathetic system. Once induced, the proptosis is not modified by death or anesthesia.

Histological changes within the orbits of exophthalmic fish consist of oedema and cellular infiltration of the areolar and fatty retrobulbar tissues, muscles, and conjunctiva. The major alteration, however, is the presence of a large amount of fluid in the retrobulbar space. Five times as much fluid was recovered from exophthalmic orbits as from normal orbits. In withdrawing the fluid, the exophthalmos immediately disappears. When the right orbit is opened to the outside by a fistula, or by inserting a drain into the retrobulbar space, and the fish are injected with pituitary extract, exophthalmos is produced only in the normal, or left orbit. The decompressed orbit does not become proptotic.

Eyeballs may be made proptotic artificially by introducing fluid into the retrobulbar space. Such proptosis disappears within 18 hours if the fluid is saline, thus showing that the orbital tissues are capable of resorbing fluid. However, if the introduced fluid is pituitary extract, the artificial exophthalmos may extend for over one week, showing a direct action of the exophthalmic factor on the orbital tissues. The ability of the orbital tissues to absorb fluid is further indicated by the fact that 1 day after instillation of pituitary extract to the right eye, the left eye shows exophthalmos, of lesser severity and duration. The natural retrobulbar fluid which forms after intraperitoneal injection of pituitary extract was collected and instilled into the right orbits of normal animals. The behavior of the right and left eye was the same as the described for pituitary extract. Assay of the retrobulbar fluid in the routine fashion disclosed the presence of exophthalmic substance, thus accounting for the above similarity of the fluid and pituitary extract when applied locally. Nonabsorbable fluids like oil maintain when instilled locally a maximal artificial exophthalmos, which seems permanent.

The mechanism of exophthalmos in Fundulus is conceived as being due to the secretion of fluid by the orbital tissues into the retrobulbar space. The accumulation of fluid exerts pressure on the posterior surface of the eyeball, thrusting it out of the orbit when the pressure overcomes the inward pull of the extraocular muscles. Over a period of

time, the retrobulbar fluid is slowly absorbed, and the orbital pressure decreases until the normal equilibrium is re-established and the eyeballs return to their normal position.

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INTERRUPTION OF OVULATION IN THE HEN BY SUBCUTANEOUSLY ADMINISTERED NON-SPECIFIC SUBSTANCES[1]

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INCIDENTAL to experiments which called for the subcutaneous injection into regularly laying hens of a dried egg white preparation, carried in saline, it was observed that lay was promptly interrupted. Autopsy of hens some 27–40 hours following injection disclosed more or less extensive atresia among follicles in the phase of rapid growth. Since the dried egg white was relatively high in avidin titre, it was thought possible that avidin might have caused the follicular atresia. The same egg white, however, was found to be effective after having been subjected to a temperature of 120°C. for 16 hours, thereby disposing of avidin as the agent responsible for the induced follicular atresia.

These unexpected findings served to remind us of the earlier account of Pearl and Surface (1914), in which it was shown that a fat-free desiccated corpus luteum substance, similarly administered, inhibited ovulation in the laying hen. Pearl and Surface attributed the action of the corpus luteum preparation to its "active principle," then not definitively characterized as progesterone. In view of the manner of its preparation, it does not now seem likely that the substance used by Pearl and Surface contained appreciable quantities of progesterone, and the later finding that progesterone, when administered subcutaneously, actually forced ovulation prematurely (Fraps and Dury, 1943) made their interpretation even more improbable.

The similarity of effect of preparations so different as desiccated egg white and a fat-free corpus luteum substance suggested that the observed interruption of ovulation might be caused by desiccated tissues generally. Preliminary tests confirmed this supposition, and further work showed that the same effect could be obtained also with subcutaneously injected proteins and peptone.

MATERIALS AND METHODS

Most of the hens available for this work were in no more than fair, but regular, production when selected for test. Rhode Island Red, White Leghorn

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[1] In the present context, the term non-specific implies only the absence of any known hormone in the preparations administered.

and cross-bred birds were used, no account being taken of possible reaction differences between the breeds. The birds were maintained in individual cages in laying batteries, with access to feed and water at all times. The batteries were under electric lights from 5:00 A.M. through 7:00 P.M. Records of lay, based on the hourly collection of eggs from 7:00 A.M. through 3:00 P.M. (Eastern Standard Time), were available for all hens both before and following injection.

Non-Specific Substances. The egg-white preparation was air dried at low temperature. The corpus luteum residue was presumably similar to that used by Pearl and Surface (1914), and was specifically described by the manufacturer to contain no known hormone.[2] The ovarian residue,[2] likewise described as free of hormone content, was prepared from ovarian tissue lacking any corpora.

The chicken tissues—breast muscle, brain and oviduct—were prepared in this laboratory. The fresh tissue was cut into small pieces which were further broken down in a Waring Blendor and spread thinly over glass plates. After drying for several days at 36°C., the material was scraped from the glass, ground in a porcelain mortar into a fine powder and finally sifted through four layers of cheesecloth. The resultant powders were at least physically comparable with similar commercial products.

The crystalline ovalbumin was prepared and kindly made available to us by Dr. E. H. McNally of this station. The ovalbumin was recrystallized 4 times and probably represents as pure a protein preparation as can be obtained by recrystallization. Casein and peptone were obtained from commercial sources.

All the preparations were taken up in 0.9 per cent saline. The usual procedure was to work up the material with a little saline, using a mortar and pestle for the purpose, until it was thoroughly wetted, after which more saline was added. It was found difficult to obtain sufficiently dilute suspensions of some preparations, in the quantities used, in the equivalent of less than 60 ml. saline per bird. When 60 ml. saline were used, 20 ml. were injected subcutaneously in the back of the neck and at right and left breast sites. Most of the substances were carried by much lesser volumes of saline in at least certain tests in each series.

In preliminary and a few other tests the hens were injected between 10:00 and 11:00 A.M. of one day and all were autopsied between 1:00 and 3:00 P.M. of the following day for direct examination of follicles in the rapid growth phase. It was soon found that follicular atresia was invariably induced in a greater or lesser number of these follicles when the various substances were injected at sufficiently high levels, and it seemed reasonable to suppose that records of lay—or of interrupted lay—would serve in general as a measure of the extent of follicular atresia. This latter procedure, with daily rectal palpation for assurance that eggs were not being held *in utero*, was followed in the majority of tests.

Only follicles in the phases of rapid growth were examined for atresia at autopsy of treated hens. The atretic follicle is described elsewhere (Rothchild and Fraps, 1945).

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[2] Corpus luteum residue and ovarian residue (free of corpora). Parke, Dayis and Company.

Observations on inhibited or delayed ovulation were necessarily based on autopsied material, since either inhibited ovulation or atresia would of course result in failure of lay. Hens due to ovulate at known times on the day following injection were accordingly autopsied at various times subsequent to the time of expected ovulation and the condition of the ovulable follicle was ascertained if ovulation had not occurred. If ovulation had taken place, the time of occurrence of the event was estimated from place of the egg in the oviduct.

Non-treated and experimental controls. As was noted earlier, practically all tests reported on here were carried out with hens in low production and these were used in large part during the season of normally declining production (June through August). Under these circumstances it was expected that, within any group of hens selected on a given date, a certain number would subsequently suffer more or less prolonged lapses in ovulatory schedules independently of any experimental treatment. If preparations were injected at sufficiently high levels, the normal incidence of such ovulatory lapses was of little moment, since ovulation was abruptly interrupted for several days or longer in a high proportion of all of the treated hens. At lower levels the action of injected materials was less drastic and objective criteria, at least semi-quantitative in nature, were required for the evaluation both of expected normal lapses and experimentally induced interruptions.

Two measures of effect were adopted. Both rested on the selection of hens which, although ovulating at low or moderate rates, exhibited during a period of 10 days or longer preceding selection (or injection) predominantly single-day intervals between clutches (single ovulations also constituting a clutch in this connection) and practically never more than a two-day interval. In a very few instances birds resuming ovulation at a good rate within the 10-day period preceding selection were also used.

A first estimate of interrupted ovulation consisted simply in calculating the percentage of hens exhibiting lapses in ovulation for 3 days or longer within the 10-day period subsequent to the day of injection or selection. With the use of records of lay directly as the source of these data, the first day following injection is, of course, excluded, since the egg laid on this day was ovulated on the day of, and usually prior to the hour of injection.

The second measure of normally occurring or induced ovulatory lapses was based on comparison of the percentage of ovulations occurring in a given group of hens during the 10-day period preceding selection or injection—ovulation by each hen each day during the period being taken as 100 per cent—with the percentage recorded for the 10-day period following injection.[3] The most convenient expression of this comparison is the quotient of the number of ovulations occurring within the 10-day period subsequent to injections divided by the number occurring in the same period preceding injection or selection (p). The quotient s/p is designated the ovulation quotient.

Applying these criteria, a comparison was made of the ovulatory behavior of several groups of hens selected on given dates exactly as were in-

[3] Percentage ovulation is approximately equivalent to ovulation frequency (Rothchild and Fraps, 1945). The latter measure of ovarian activity can be applied only to data based on definitive ovulation cycles, which of course are lacking when ovulation is completely interrupted.

jected hens, but receiving no treatment whatever. The results are given in table 1, together with similar data on one group of hens receiving saline alone (60 ml.) and a second group injected with the same quantity of distilled water. As might be expected, there is a reasonably good agreement between the two indices. It is notable that among the non-treated controls those groups selected on June 15 and July 31 show a decrease in the value of the ovulation ratio (s/p) of the same order found following the injection of saline or distilled water. The use of a relatively large volume of saline as a carrier

TABLE 1. COMPARISON OF OVULATORY BEHAVIOR OF NON-INJECTED HENS BEFORE AND FOLLOWING DATE OF SELECTION AS TYPICAL TEST BIRDS WITH SIMILARLY SELECTED HENS RECEIVING 60 ML. SALINE (0.9%) OR 60 ML. DISTILLED WATER

Treatment and dates	Hens selected for test	Hens exhibiting interrupted ovulation		Percentage ovulation		Ovulation ratio
				Before selection (p)	Following selection (s)	(s/p)
	No.	*No.*	%	%	%	
Non-injected (May 31)	15	3	20	70	63	0.90
Non-injected (June 15)	15	5	33	67	47	0.72
Non-injected (July 31)	11	4	36	62	43	0.69
Non-injected (August 31)	15	4	27	59	49	0.86
Saline (June-July)	14	4	29	72	48	0.67
Distilled Water (July)	9	4	44	68	47	0.69

seems therefore justified, although some small effect may follow its administration.

On the basis of the above data, we have attributed only a questionable effect to an injected substance if less than about 50 per cent of the treated hens show ovulatory lapses of 3 days or longer, or if the ovulation quotient, s/p, is appreciably greater than 0.50.

RESULTS

Representative results, based on the injection of ovalbumin and peptone, are recorded in table 2. The effect of ovalbumin, 0.25 gm. per hen, is pronounced, but not extreme. The action of peptone at the 1.0 gm. level is fairly drastic, although it was administered in only 10 ml. saline per bird.

All hens recorded in table 2 were selected for ovulation of the first follicle of the oncoming clutch the morning following injection. The interval from injection to average time of expected ovulation is about 20 hours. The results indicate that in at least certain hens the ovulatory sequence may be completely disrupted within this relatively brief interval.

Following injection some hens failed to ovulate during the 15 days of record (table 2), and some of these for a month or longer. It is doubtful that ovulatory lapses of more than two or three weeks can be attributed entirely to injected materials (Pearl and Surface, 1914).

As typical of dose-response relations, the action of dried egg white injected at various dosages and in varying quantities of saline is summarized in table 3. When administered in 60 ml. saline, there is a regular decrease in effect with decreasing dosage over the range of 10–1.25 gm. per hen. The injection of either 1.0 or 2.0 gm. egg white in relatively small quantities of saline is of doubtful effect. Absorption

TABLE 2. TYPICAL RECORDS OF LAY OF HENS PRECEDING AND FOLLOWING SUBCUTANEOUS INJECTION OF NON-SPECIFIC SUBSTANCES

Hen No.	Lay preceding injection		Lay following injection
	Ovalbumin, 0.25 gm. in 60 ml. saline per hen		
I2431	+ − + + − + − + − + +	+	− + − + − + + − + + − + +
K5153	+ − + − − + + + − + − +	+	− − − − − − − − − − − − −
N3621	+ − + + − + − + + − + +	+	+ + − + − − − − + − − − −
Z6121	− − − − − − − + − + − +	+	− − − − − − − − − − + − + +
I2871	+ − + − + + − + − + − +	+	− − + − − − − − − − − − −
K5071	− + − + + − + − + − + +	+	− + − + − + + − − − − − −
	Peptone, 1.0 gm. in 10 ml. saline per hen		
N2889	+ − − + − + − + − + − −	+	− − − − − − − − − − − − −
514	− − + − + − + − + − + +	+	− − + − − − − − − − − + + − −
L8728	− + + − + − + + − − + −	+	− + − − − − − − − − − − − −
L8863	+ − + − + − + + + − + +	+	− − − − − − − − − − − − − −
L36	+ − + − + + − + − + − + −	+	− + − − − − − + − − + − − + −

The plus sign (+) designates lay on a given day, the minus sign (−) absence of lay The blocked column is the day upon which the birds were injected.

TABLE 3. EFFECT OF SUBCUTANEOUSLY INJECTED DRIED EGG WHITE ON OVULATORY BEHAVIOR

Quantity injected	Volume of saline	Hens injected	Hens exhibiting interrupted ovulation		Percentage ovulation		Ovulation ratio
					Before injection (p)	Following injection (s)	(s/p)
gm.	*ml.*	*No.*	*No.*	*%*	*%*	*%*	
10.00	60	4	4	100	73	0	0
5.00	60	6	6	100	65	25	0.38
2.50	60	10	7	70	68	29	0.43
1.25	60	20	13	65	63	32	0.51
2.00	12	6	4	67	60	42	0.70
1.00	6	11	5	45	65	45	0.70

phenomena undoubtedly play a role in accounting for the greater effectiveness of comparable quantities of materials in greater volumes of saline, but it is difficult to evaluate the variables critically on the basis of present results.

On the basis of data comparable to those given in detail in tables 2 and 3, the effects of six different preparations, injected at designated dosages, are summarized in table 4 with respect to percentage of hens exhibiting interruption of ovulation (3-day lapse or longer) and value of the ovulation ratio calculated by comparison of ovulations during the usual 10-day periods preceding and succeeding injection. It need

hardly be said that no quantitative comparison is implied in the data of this table, a point sufficiently emphasized by the range of values of the ovulation ratio (s/p). All the substances injected at indicated levels caused moderate to severe interruption of ovulation. The corpus luteum and ovarian residues, injected in 20 ml. saline, would probably prove equally effective at lower dosages if administered in 60 ml. of the same carrier (cf. table 3).

The effectiveness of tissues of chicken origin—muscle, brain, and oviduct—was demonstrated on the basis of autopsy findings and thus cannot be compared directly with the data of table 4. All induced atresia or suppressed ovulation following injection at the 2.0 gm. level (20 ml. saline), the muscle preparation being perhaps somewhat more

TABLE 4. EFFECT OF VARIOUS NON-SPECIFIC SUBSTANCES ON THE INTERRUPTION OF OVULATION AT DESIGNATED DOSAGE LEVELS

Substance administered	Quantity injected	Volume of saline	Hens injected	Hens exhibiting interrupted ovulation	Ovulation ratio following injection
	gm.	*ml.*	*No.*	%	
Corpus luteum residue	2.00	20	9	100	0.28
Ovarian residue (free of corpora)	2.00	20	8	88	0.28
Egg white	1.25	60	20	64	0.51
Ovalbumin, crystalline	0.25	60	6	83	0.36
Casein	1.00	20	6	100	0.03
Peptone	1.00	10	10	100	0.14

effective than the other preparations. It is of particular interest that desiccated tissue preparations of chicken origin are effective in the hen of the same species, indicating that the reaction in question is not dependent upon the injection of heterologous tissue preparations. The action of egg white and ovalbumin points to the same conclusion, but in the absence of other data these substances might be considered more narrowly as reproductive products, along with corpus luteum and ovarian residues of mammalian origin.

Suppressed ovulation. In several groups of hens placed on test for incidence of atresia at autopsy some 27 hours following injection, only intact follicles were found. Most of these hens were selected for expected ovulation of the first follicle of the oncoming clutch sequency between 4:00 and 7:00 A.M. of the day following injection, and were autopsied between 1:00 and 3:00 P.M. of the day of expected ovulation. Ovulation was therefore delayed 6 to 11 hours beyond its expected time without evidence of the onset of atresia. The incidence of suppressed ovulation was greater at relatively low doses, sufficiently high doses imposing prompt and extensive atresia. Although our observations in this connection are limited, no evidence indicated that the suppression of ovulation was peculiar to any of the various substances tested.

DISCUSSION

The number of desiccated tissue preparations, proteins, and protein derivatives capable of interrupting the ovulatory sequence of the regularly ovulating hen might in all probability be extended indefinitely. The date recorded in this paper seem adequate, however, to dispose of any notion that the effects in question are peculiar to hormonally potent preparations. As was noted earlier, the fat-free corpus luteum preparation used by Pearl and Surface (1914) almost certainly belongs to this class of substances which, from an endocrinological point of view, is wholly non-specific.[4]

Since at least two proteins (ovalbumin and casein) and one secondary derivative of protein (peptone) bring about the same effects as do the dried tissues, it seems most probable that the action of the desiccated tissue preparations can be attributed to their content of proteins or protein derivatives. In any event, there is no occasion to postulate, for any of the several tissue preparations used in our tests, unique or specific properties.

The interruption of ovulation by non-specific substances might be accounted for either as a direct effect on the ovarian follicle, or as an indirect effect consequent upon disruption of the normal regulatory controls over follicular growth, maturation and ovulation. The latter alternative seems more probable in view of the apparently suppressed ovulation of follicles which are at least macroscopically intact (non-atretic) when observed hours later than the time of normally expected ovulation. An obvious explanation of suppressed ovulation is that the injected substances partially or completely block the episodic release of ovulation-inducing gonadotrophin by the anterior pituitary. The more drastic action of the same substances at higher doses might on similar grounds be attributed to a more complete and prolonged block of gonadotrophin secretion involving perhaps the suppressed output of follicle stimulating hormone as well as of the hormone effecting normally timed ovulation. The actual mechanism involved may not, however, prove so simple, and alternative possibilities are not far to seek. Only one of these may be noted briefly.

Numerous substances, when injected with anterior pituitary preparations or extracts, augment the effectiveness of pituitary gonadotrophin (Van Dyke, 1939). It may be noted specifically that both casein and egg-albumin are effective potentiating substances when injected in combination with gonadotrophins (Saunders and Cole, 1936), and both promptly interrupt ovulation when injected alone

[4] It is perhaps a matter of historical interest to acknowledge that progesterone, the "active principle" to which Pearl and Surface attributed their results, does in fact induce follicular atresia with consequent interruption of ovulation in the hen (Schoeller and Gehrke, 1933) and in the pigeon (Dunham and Riddle, 1942). Under appropriate conditions of dosage and timing, however, progesterone is a highly effective ovulation-inducing agent in the hen (Fraps and Dury, 1943), atresia or interruption of ovulation occurring secondarily.

into the regularly laying hen, as is shown in this paper. Additional evidence has recently been adduced in favor of the view that the augmentation effect is due to adsorption of the gonadotrophin by the simultaneously injected agent, with subsequent slow but prolonged release of the gonadotrophin (McShan and Meyer, 1945). A conjecture of possible interest is that the non-specific substances interrupting ovulation do so by a comparable adsorption of endogenous gonadotrophins. If gonadotrophins in the blood stream were in fact so adsorbed, we might reasonably expect first the failure of occurrence of ovulation, and with the continued immobilization of endogenous gonadotrophins, the onset of follicular atresia.

The foregoing speculations perhaps merely emphasize the need for some basis in fact of the actual mechanism through which follicular atresia (or inhibited ovulation) is mediated. Without supposing that all agencies act through the same mechanism, it seems likely that most if not all may involve the anterior pituitary gland directly or indirectly. If so, clarification of the manner in which the anterior pituitary gland (or its gonadotrophins) may be implicated in the causation of atresia might be expected also to enhance our knowledge of the conditions favoring maintenance of ovarian function at reproductive levels.

SUMMARY

The subcutaneous injection, in saline, of various non-specific (i.e., hormone-free) substances inhibits or interrupts ovulation in the regularly laying hen. Effective preparations include desiccated tissues (corpus luteum and ovarian residues of the cow; brain, muscle and oviduct of the chicken), whole dried egg white, proteins (ovalbumin and casein) and peptone. At relatively high doses all of these substances bring about prompt and extensive follicular atresia, with cessation of ovulation. Control injections of saline and distilled water were of little or no effect. The effectiveness of ovalbumin, casein and peptone indicates that the action of the dried tissue preparations and egg white may be attributed to their content of proteins or protein derivatives. Following the administration of some preparations at relatively low doses, ovulation was inhibited for at least 6 to 11 hours beyond the time of its normal occurrence without evidence of the onset of atresia, suggesting that the non-specific substances may act through the anterior pituitary gland or its gonadotrophins.

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THE RELATION BETWEEN OVULATION FREQUENCY AND THE INCIDENCE OF FOLLICULAR ATRESIA FOLLOWING SURGICAL OPERATIONS IN THE DOMESTIC HEN

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INTRODUCTION

THE REPORTS in the literature dealing with follicular atresia in the ovary of the bird have been largely confined to morphological descriptions of the process (Davis, 1942; Pearl and Boring, 1918; Dubuisson, 1906) and to mention of some of the agents which bring it about. Examples of these are environmental disturbances, (Stieve, 1918); injections of various steroids (Dunham and Riddle, 1942), sub-ovulatory doses of pituitary preparations (Fraps and Dury, 1942), dried tissue preparations and egg white, proteins, and protein derivatives (Fraps and Neher, 1945); and operations on the ovary (Rothchild and Fraps, 1944). In none of these studies, however, was the incidence of atresia shown to be related to any of the variables within the population of birds treated with one of these agents. The present communication will attempt to do this, and will show that one of the most important factors which determines the incidence of atresia after abdominal operations is the birds' frequency of ovulation preceding operation, and that the incidence of atresia varies in inverse proportion to this factor.

This relationship was discovered in analyzing the ovulatory behavior of a group of domestic hens which had been subjected to removal of the most recently ruptured follicle. (This study was a continuation of one in which it was shown that removal of the ruptured follicle resulted in delayed lays (Rothchild and Fraps, 1944).) The oldest preovulatory follicle in these birds either underwent atresia, showed a delay in its time of ovulation, or ovulated at the expected normal time. In general, atresia occurred most frequently in birds with the lowest ovulation frequencies, delayed ovulations most frequently in birds with low, or intermediate ovulation frequencies, while ovulations at the normally expected time were most frequent in birds with the highest ovulation frequencies. Further studies showed that the abdominal operation itself, rather than the removal of the rup-

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tured follicle, was primarily responsible for the occurrence of atresia, but that, in sharp contrast to the effects of abdominal operations, operations of apparently similar extent and severity on the breast muscle were practically ineffective in inducing atresia.

The data presented below will demonstrate the inverse relation between the incidence of atresia and the frequency of ovulation, will show the relative effects of different operations and will estimate the role of factors other than ovulation frequency in determining the incidence of atresia, and the way in which these factors may affect the relation between the incidence of atresia and ovulation frequency.

The process of atresia in the hen's ovary. The first stage of atresia which is macroscopically visible is characterized by the appearance of a number of very small blebs upon the surface of a follicle. These are apparently formed by the collection of yolk fluid between the inner and outer walls of the follicle; the immediately determining cause of this is a breakdown of the inner wall of the follicle and of the vitelline membrane of the yolk. As atresia proceeds, the blebs enlarge and coalesce, until the whole surface of the follicle is covered with them up to but not usually including the stigma. Eventually all the yolk fluid is lost, either by continued leakage out of the follicle when the outer wall breaks down, or by absorption into the blood stream. The appearance of the follicle in advanced stages varies somewhat. In most cases it becomes considerably shrunken, the outer wall taking on a baggy appearance as the follicle shrinks in size, and the yolk fluid disappears. The central cavity becomes occupied either with very thin watery yolk, with yolk mixed with serous fluid, or with the yolk entirely replaced by serous fluid. In some cases the color of the follicle is a chocolate or purplish brown, due to the mixture of broken down blood elements with the yolk. Eventually the whole follicle disappears macroscopically by resorption. (See also the description of follicular atresia in the pigeon by Dunham and Riddle, 1942).

Although it is not at all difficult to distinguish between early and advanced stages of atresia, we are in some doubt at the present time of the exact time relations involved in the process during the first 48–72 hours. However, this has been of secondary importance for these studies.

The clutch, the cycle and ovulation frequency. In order to facilitate understanding of the procedures used in selecting birds for operation, following them afterwards, and classifying them into those showing and not showing atresia, as well as to make clear the meaning of ovulation frequency, the following brief description of ovulatory and lay behavior in the hen is offered.

The domestic hen in good production lays an egg a day over a number of successive days, misses lay usually on but a single day, then approximately repeats the cycle. The series of eggs laid on successive days is called a clutch; its size may vary from 1 egg to more

than 50, although the majority of clutches laid by most birds do not vary much beyond 1 to 5 eggs. In general, clutches follow one another with pauses of a single day of non-lay between them, although pauses of 2 or even 3 days sometimes occur regularly between successive clutches in birds in low production.

An interval of roughly 27 hours intervenes between ovulation and lay of a particular egg. Within a clutch, the interval between lay of one egg and ovulation of the next is about 30 minutes. Between lay of the terminal egg of one clutch and ovulation of the first egg of the next clutch an interval of about 18 hours intervenes. The first egg of a clutch is ovulated early in the day (about 5:00 A.M. EST under conditions in this laboratory), and the terminal egg comparatively late (between 11:00 A.M. and 2:00 P.M. EST). We will refer in the following pages to the first egg of the clutch as C_1, the successive intraclutch eggs as C_s (Fraps and Dury, 1943), and the terminal egg of the clutch as C_t. For fuller details concerning clutch types and behavior, the papers of Atwood (1929), Heywang (1938), and Warren and Scott (1935) should be consulted. It should be clear from this that a bird's record of lay, in the vast majority of cases, exactly corresponds to its record of ovulation, the latter record simply preceding the former record by one day.

We have used the term ovulation frequency to mean simply the ratio of clutch length over cycle length, the latter being defined as the period of time, in days, from the lay of the first or last egg of one clutch (or its ovulation), up to, but not including, the corresponding event in the next clutch. In this study no birds were used in which the interval between successive clutches exceeded three days. The ovulation frequencies were calculated for the period of roughly 15 days preceding the operation. This time period included two or more cycles for each bird, and varied from bird to bird because it was made to conform to the peculiarities of each bird's cycle. The shortest time period used was 9 days, and the longest 25, but in 84% of the birds it varied between only 13 and 16 days, and in 95% between 12 and 17 days.

METHODS AND MATERIALS

A. Operative Procedures.

1. *Removal of a ruptured follicle.* Birds were operated on for removal of either the youngest ruptured follicle (R_1), the next older one (R_2), or one older than either of the first two (R_3 or older).[1] The procedure followed was the same in all cases.

The birds were anesthetized with Nembutal (Pentobarbital Sodium, Abbott) by intravenous injection. The amount required was roughly be-

[1] The ruptured follicle regresses very rapidly after its formation, losing more than one-half its original weight in the first 48 hours. Thus, while it is comparatively easy to recognize the two youngest ruptured follicles, and to distinguish between them, it is often very difficult to distinguish by sight alone between the R_3 follicle and those older than it.

tween 0.25 and 0.35 cc. (6% solution) per kilo of body weight. The left side of the abdomen was plucked clean, and the bird tied to the operating table by the wings and right leg, the left leg being pulled out of the operating field by means of a cheesecloth loop. This exposed a triangular area bounded by the posterior border of the ribs, the lateral border of the pelvic girdle, and the sternal-cloacal line, the latter being the base of the triangle. The ovary lies just a little anterior to the apex of the triangle. An incision was made in the skin extending from the posterior-lateral edge of the ischium to the apex of the triangle; incisions in the three layers of abdominal muscle were then made in the same direction but not quite to the same extent and the peritoneum was exposed. The latter was cut starting with the most posterior portion that could be picked up without including the fat layer, and extending forward about 3 inches. The thin membrane which covers the ovary and oviduct was then torn as far from the ovary as possible, and the ovary exposed. The whole operation to this point lasted approximately 3 minutes.

The handling of the ovary to expose and remove the ruptured follicle desired was done as carefully as possible, using a silver-plated teaspoon as a retractor. As soon as the ruptured follicle was seen it was picked up with a pair of blunt forceps, and cut at its base with a pair of scissors. Immediately after cutting, a small cotton sponge was splaced over the cut stump, the ovarian membrane replaced over the ovary, and the edges of the wound roughly approximated, while the needles required for sewing the muscle and skin walls were threaded. The amount of time consumed in threading the needles was usually sufficient to control the bleeding, after which the sponge was removed, the membrane replaced as well as possible, and the muscle and skin walls sewn separately, by continuous suture, with cotton thread.

2. *Sham ovarian operations.* These were done in exactly the same manner as those just described, except that no portion of the ovary was removed.

3. *Sham operations without handling ovary.* The operation was carried out identically on the right side of the abdomen. The ovary was not seen or handled in any way.

4. *Operations on the breast muscle.* The birds were anesthetized and fixed to the operating table in the same way as for removal of a ruptured follicle. The feathers over the left breast were then plucked clean. An incision was made in the skin extending anteriorly about 5 or 6 inches from the posterior tip of the sternum, and parallel with the ventral edge of the sternum. The superficial pectoral muscle was cut with a pair of scissors, starting close to the caudal edge of the skin incision, and extending forward about 4 inches. The opening was widened with a pair of blunt forceps, and the muscle separated cleanly from the deep pectoral. Bleeding was induced by tearing some of the blood vessels which lie between the two muscle layers. After allowing time for clotting to be completed, the incision was cleaned out with a small cotton sponge, the muscle and skin sewn separately with cotton thread, and the birds treated from this point on exactly the same as the other operated birds.

General. Where possible, observations were taken at the time of operation of any injury which might have been inflicted on the ovary, the extent of bleeding, and in general, on the care with which the operation was carried out. The duration of the operation rarely exceeded 10–15 minutes. Aseptic procedures were not used, except in a very few birds. Cleanliness was observed

by washing and drying the instruments between operations. Very few birds developed more than mild and localized wound infections.

Following operation, the birds were placed on the floor on clean newspaper, and allowed to remain there until they stood up of their own accord. They were then replaced in their cages under the same conditions as those preceding operation.

B. Maintenance of Birds, and Selection for Operation.

All the hens used in this study were housed in individual cages in laying batteries. Collections of eggs were made hourly on the hour every day, including Sunday, from 7:00 A.M. to 3:00 P.M. (EST) with an additional collection at 7:00 P.M. The birds were maintained on at least a 14 hour light-day all year round by lights in the battery rooms, automatically turned on at 5:00 A.M. and off at 7:00 P.M. every day. They had unlimited access to a laying mash and water.

With only two exceptions, the birds in these studies were selected for operation only when an egg was present in the oviduct. The two exceptions were birds selected within a few hours after the lay of a C_t egg. In 283 birds the oviducal egg had been ovulated 14 hours or less before operation, and in 5 birds between 25 and 29 hours before operation. Most of the birds carried C_1 oviducal eggs, but some carrying C_s and C_t eggs were also used.

C. Post-Operative Procedures to Determine the Occurrence of Atresia.

Following operation the treatment of the birds varied somewhat depending upon the behavior of the oldest maturing follicle (M_1 follicle). Two courses were followed: the birds were either 1) kept alive for an indefinite period and the behavior of the follicle followed by cloacal palpations; or 2) were killed on the first, second, or third day after operation, and the behavior of the follicle judged by actual observations of the ovary and oviducal contents. In general, those birds in which a freshly ovulated oviducal egg could be palpated on the day after operation were kept alive, while all those in which such an egg could not be palpated on the first and second day after operation, and most of those in which palpation was negative on the first day, but positive on the second day were killed on the second day after operation. There were some exceptions to this rule. Thus, 12 birds were killed on the first day after operation, 10 of which gave negative cloacal palpations on this day and 2 of which showed the presence of a freshly ovulated yolk. Thirteen birds were killed on the third day after operation, 2 of which showed the presence of a freshly ovulated yolk on the first day after operation, 7 of which gave a positive palpation on the second day but not the first day after operation, and 4 of which were negative on both days. Nineteen of the 59 birds which were kept alive gave positive palpations on the second, but not on the first day after operation. Twelve of the 204 birds which were killed on the second day after operation, gave positive cloacal palpations on the first day after operation.

In all cases the classification of a bird as one showing atresia depended on actual observation of the M_1 follicle at *autopsy*. On the other hand, the classification of a bird as one not showing atresia depended either on actual observation of this follicle at autopsy, or on the fact that cloacal palpations showed that it had ovulated on the first or second day after operation. This

was done, except where noted otherwise, regardless of whether other maturing follicles than the M_1 were atretic at autopsy, or in the case of the birds which were kept alive, showed by their lay records evidence of subsequently undergoing atresia.

D. Autopsy.

At autopsy the following points were noted: body weight, degree of healing of wound, and presence of infection in it; presence and extent of adhesions, and of yolk fluid in the body cavity; weight of the oviduct, ovary, and maturing follicles in the rapid growth phase, number and weight of the atretic follicles and degree of atresia; weight of the ruptured follicles, and if present, of the oviducal egg, and its yolk, and measurement of its position in the oviduct.

No birds were included in the summaries which showed evidence of a faulty operation (poor healing, heavy infection, numerous adhesions, etc.), or in which there was doubt as to the exact sequence of events.

E. Kinds of Birds, etc.

Four different breeds or breed types of birds were used. These were Rhode Island Red, (RIR), White Leghorns, (WL), crosses between RIR and WL, (WLX), (in most cases F_1 RIR♂ × WL♀), and F_1 crosses between RIR♂ and Barred Plymouth Rock♀ (RIRX). They were of several different ages, divided for convenience into three groups, i.e., less than 1 year to 1 year, older than 1 year to 2 years, and older than 2 years. The operations were performed throughout the year (April '44 to April '45) except during the months of August and September. The ovulation frequencies of the birds used varied from 0.43 to 0.93. A total of 288 birds was used.

PRESENTATION OF RESULTS

A. Relation between Ovulation Frequency and the Incidence of Atresia and the Relative Effects of Different Operations.

1. Abdominal operations. Removal of the most recently ruptured Follicle. One hundred and eleven birds were operated on for removal of the R_1 ruptured follicle. The separation of these birds into groups with different ranges of ovulation frequencies, and the percentage of birds showing atresia in each group are shown in the first row of Table 1. It can be seen that in the group of birds with ovulation frequencies between 0.43 and 0.60, the incidence of atresia was 57%, and that this value fell to 5.6% in the group with ovulation frequencies between 0.81 and 0.93. *Removal of the next to the youngest ruptured follicle.* Fifty-one birds were subjected to this operation. In this group of birds there was a drop in the incidence of atresia from 58% in the 0.43–0.60 ovulation frequency group to 17% in the 0.81–0.93 group (2nd row, Table 1). *Removal of a ruptured follicle older than either of the first two.* In the 39 birds which were subjected to this operation, the incidence of atresia was of the same general magnitude as it was in the preceding two groups, and bore approximately the same relation-

ship to the frequency of ovulation (3rd row, Table 1). *Sham ovarian operations and operations without handling of the ovary.* In those birds subjected to sham ovarian operations (35 birds), and to operations without handling of the ovary, (15 birds), (4th and 5th rows, Table 1), there was the same relationship between the incidence of atresia and the frequency of ovulation as has already been noted. There was a

TABLE 1. THE INVERSE RELATION BETWEEN OVULATION FREQUENCY PRECEDING OPERATION AND THE INCIDENCE OF ATRESIA AFTER IT
(For details see text)

	Ovulation frequency groups							
	0.43–0.60		0.61–0.70		0.71–0.80		0.81–0.93	
Type of operation	No. of birds	% showing atresia	No. of birds	% showing atresia	No. of birds	% showing atresia	No. of birds	% showing atresia
Removal of youngest ruptured follicle	14	57% (8)[1]	29	45% (13)	50	28% (14)	18	5.6% (1)
Removal of next older ruptured follicle	12	58% (7)	15	53% (8)	18	33% (6)	6	17% (1)
Removal of ruptured follicle older than either of first two	9	55% (5)	15	40% (6)	12	33% (4)	3	0%
Sham ovarian operation	17	76% (13)	9	44% (4)	5	20% (1)	4	0%
Abdominal operation. Ovary not handled	10	80% (8)	5	60% (3)	—	—	—	—
Breast muscle operations	17	12% (2)	13	0%	6	0%	1	0%
ALL ABDOMINALLY OPERATED BIRDS	62	66.1%	73	46.5%	85	29.4%	31	6.4%

[1] This and all other figures in parentheses represent the number of birds showing atresia in each group.

difference, which is probably statistically insignificant, between the incidence of atresia in the birds with low ovulation frequencies subjected to the sham operations, and those subjected to removal of a ruptured follicle.

When all the birds described above were grouped together according to differences in ovulation frequency (bottom row, Table 1), an approximate 10-fold difference in the incidence of atresia appeared between the group with ovulation frequencies of 0.43–0.60 (average 0.54, incidence of atresia 66.1%) and that with ovulation frequencies of 0.81–0.93 (average 0.85, incidence of atresia 6.4%). The inverse relation is clearly brought out in Figure 1.

It is obvious that the induction of atresia is not a specific effect of

removal of the ruptured follicle, or even of handling the ovary. That it is closely associated with the effects of an abdominal operation is shown by comparison with the results obtained with breast muscle operations.

2. Breast muscle operations. A total of 37 birds was used (6th row,

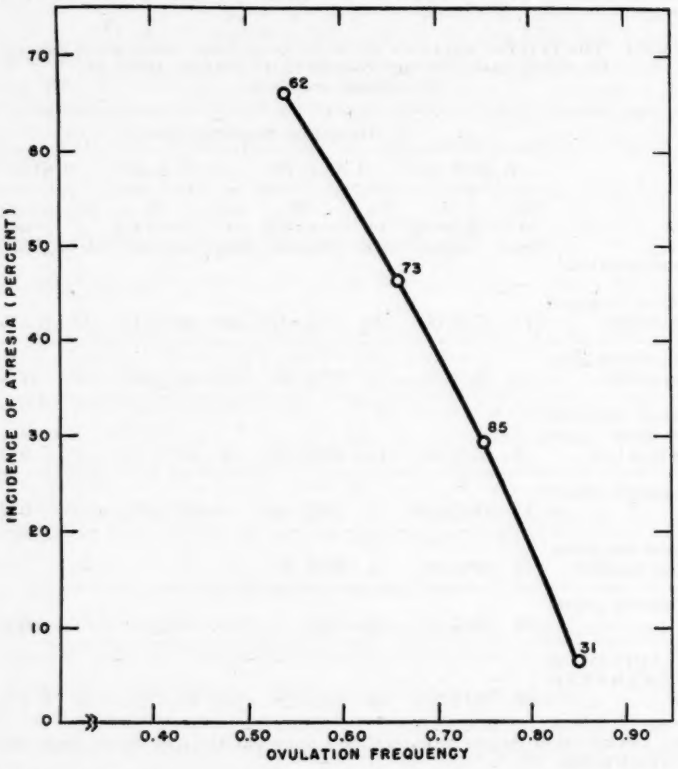

FIG. 1. *The relation between the incidence of atresia following abdominal operations and the frequency of ovulation preceding the operation.* The number above each point refers to the group of birds from which the average ovulation frequency was derived.

Table 1). The ovulation frequencies in this group ranged from 0.46 to 0.82, but most of the birds (30) fell within the range of 0.46 to 0.70. As can be seen from Table 1, only 2 birds went into atresia following this operation.

B. Relation of Incidence of Atresia to Other Factors.

The relations between the incidence of atresia and age, breed, clutch position of the oviducal egg, time after ovulation that operation was performed, season, and change in body weight after operation are

TABLE 2. THE RELATION BETWEEN INCIDENCE OF ATRESIA AND AGE, BREED, SEASON WHEN OPERATED ON, POSITION IN CLUTCH OF OVIDUCAL EGG AT TIME OF OPERATION, TIME AFTER OVULATION THAT OPERATION WAS PERFORMED AND CHANGE IN BODY WEIGHT AFTER OPERATION. FOR DETAILS SEE TEXT, PAGES 419–420.

		All birds		Ovulation frequency groups							
				0.43–0.60		0.61–0.70		0.71–0.80		0.81–0.93	
		Total no.	% showing atresia	Total no. birds	% showing atresia	Total no. birds	% showing atresia	Total no. birds	% showing atresia	Total no. birds	% showing atresia
Season	Dec.-Jan.-Feb.	119	39% (47)[1]	32	59% (19)	39	36% (14)	38	34% (13)	10	10% (1)
	Mar.-Apr.-May	81	49% (40)	26	77% (20)	21	62% (13)	25	28% (7)	9	0% (0)
	June-July	21	43% (9)	0	—	7	86% (6)	12	25% (3)	2	0% (0)
	Oct.-Nov.	30	20% (6)	4	50% (2)	6	17% (1)	10	20% (2)	10	10% (1)
Age	1 year or less	73	48% (35)	12	92% (11)	24	71% (17)	25	20% (5)	12	17% (2)
	1 year to 2 year	130	38% (49)	40	58% (23)	35	29% (10)	39	41% (16)	16	0% (0)
	more than 2 years	48	38% (18)	10	70% (7)	14	50% (7)	21	19% (4)	3	0% (0)
Breed	RIR	103	38% (39)	27	59% (16)	28	50% (14)	36	25% (9)	12	0% (0)
	WL	61	59% (36)	17	76% (13)	21	76% (16)	19	37% (7)	4	0% (0)
	WLX	56	32% (18)	14	64% (9)	19	16% (3)	19	32% (6)	4	0% (0)
	RIRX	31	29% (9)	4	75% (3)	5	20% (1)	11	27% (3)	11	18% (2)
Position in clutch of oviducal egg	C_1	192	45% (87)	60	67% (40)	57	47% (27)	57	32% (18)	18	11% (2)
	C_2	22	5% (1)	0	—	3	0% (0)	7	14% (1)	12	0% (0)
	C_3	37	38% (14)	2	50% (1)	13	54% (7)	21	29% (6)	1	0% (0)
Hours after ovulation	0– 2 hrs.	28	21% (6)	1	100% (1)	6	33% (2)	13	23% (3)	8	0% (0)
	3– 5 hrs.	128	42% (54)	31	61% (19)	33	55% (18)	48	31% (15)	16	13% (2)
	6– 8 hrs.	68	38% (26)	21	67% (14)	22	32% (7)	21	24% (5)	4	0% (0)
	9–11 hrs.	21	57% (12)	9	78% (7)	6	50% (3)	3	67% (2)	3	0% (0)
% change in body weight	+ 3% to − 2%	61	23% (14)	11	36% (4)	21	29% (6)	21	14% (3)	8	13% (1)
	− 3% to − 5%	63	41% (26)	16	50% (8)	17	47% (8)	19	47% (9)	11	9% (1)
	− 6% to − 7%	29	55% (16)	9	78% (7)	11	55% (6)	7	43% (3)	2	0% (0)
	− 8% to − 9%	15	87% (13)	5	100% (5)	6	100% (6)	3	67% (2)	1	0% (0)
	−10% to −13%	6	83% (5)	4	100% (4)	1	100% (1)	1	0% (0)	0	—

[1] This and all other figures in parentheses represent the *number* of birds showing atresia in each group.

shown in Table 2. The birds included in this table are only those subjected to an abdominal operation. The total number of 251 was available for the study of the effect of season, age, breed and clutch type of the oviducal egg. Six birds operated on between 14 and 29 hours

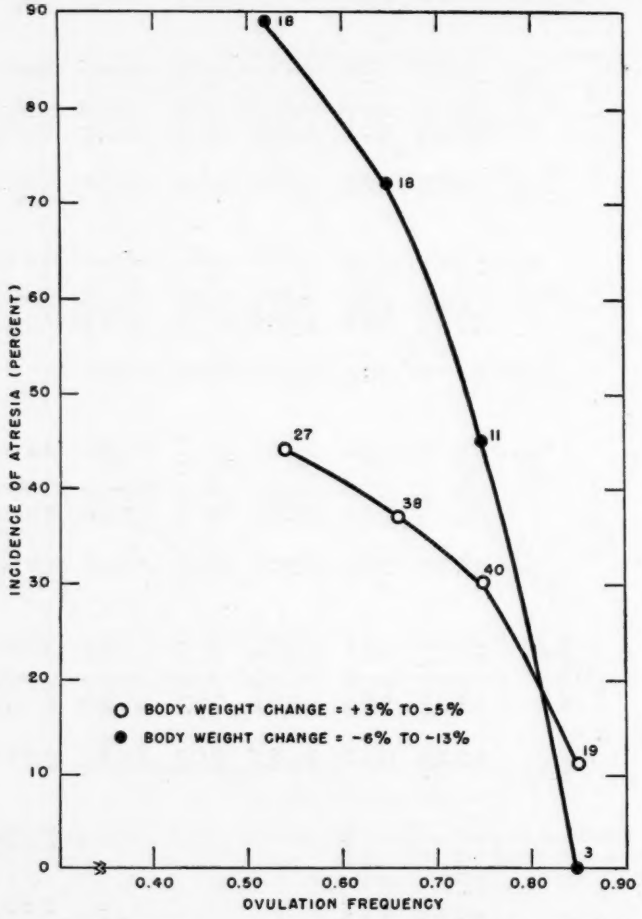

FIG. 2. *The effect of differences in weight loss on the relation between the incidence of atresia following operation and the frequency of ovulation preceding it.* The number above each point refers to the group of birds from which the average ovulation frequency was derived.

after ovulation were left out of the group broken down according to time after ovulation, since there were not enough of these falling into any one group for the results to have any meaning. For the effect of body weight change, data were available on only 174 birds. While these for the most part do not include the non-autopsied birds, the

ones which are included are a fairly good sample of the group as a whole.

It is clear from this table that in general, whatever differences there are in the incidence of atresia in relation to any of these factors can be traced to primary differences in ovulation frequency.

However, a clear direct relation can be seen between the extent of body weight loss and the incidence of atresia, and it raises the question of whether or not the inverse relation between the incidence of atresia and ovulation frequency is an artifact, primarily dependent for its existence on the occurrence of greater weight losses in the low ovulation frequency groups.

In Figure 2 is plotted the relation between ovulation frequency and the incidence of atresia in each of two separate groups of birds,—

TABLE 3. THE RELATION BETWEEN OVULATION FREQUENCY AND THE INCIDENCE OF ATRESIA IN TWO GROUPS OF BIRDS DIVIDED ACCORDING TO THE RANGE OF BODY WEIGHT CHANGE EXPERIENCED. THE AVERAGE BODY WEIGHT CHANGE FOR EACH OVULATION FREQUENCY GROUP IS PRESENTED HERE. SEE ALSO FIGURE 2.

Ovulation frequency (range)	Group A—Range of body weight change = +3 to −5%				Group B—Range of body weight change = −6 to −13%			
	Average body weight change				Average body weight change			
	All birds	Birds showing atresia	Birds not showing atresia	% showing atresia	All birds	Birds showing atresia	Birds not showing atresia	% showing atresia
0.43–0.60	−2.63%	−2.83%	−2.47%	44%	−8.11%	−8.31%	−6.50%	89%
0.61–0.70	−1.92%	−2.07%	−1.82%	37%	−7.22%	−7.71%	−6.00%	72%
0.71–0.80	−2.33%	−3.25%	−1.93%	30%	−7.55%	−7.40%	−7.67%	45%
0.81–0.93	−2.16%	−3.50%	−2.00%	11%	−7.00%	—	−7.00%	0%

those which sustained body weight changes of between +3% to −5%, and those which lost between 6% to 13% of their body weight following operation. The inverse relation between ovulation frequency and the incidence of atresia is evident in both groups, although the incidence of atresia is greater in the high weight loss group.

The average weight loss differences within each of these two groups of birds are very small (Table 3), and do not account for the differences in the incidence of atresia. This is brought out by the following comparison. Weight loss exerted its maximum effect in the group of birds with ovulation frequencies between 0.43 and 0.60 (see Figure 2). In this group those birds with an average weight loss of 2.63% showed 44% atresia, while those with an 8.11% average weight loss showed 89% atresia. Thus, a more than 300% increase in weight loss resulted in only a 100% increase in the incidence of atresia. But within the group of birds with body weight changes of from +3% to −5% there was a 400% difference in the incidence of atresia as the ovulation frequency changed, (44% in the 0.43–0.60 group to 11% in the 0.81–0.93 group), while the greatest difference in average weight loss was

only 21% (−2.63% to −2.16%). The same relation can be seen to hold in the high weight loss group (Table 3).

Finally, comparison of the birds abdominally operated on with those subjected to breast muscle operations indicate that weight loss is not the factor responsible for the induction of atresia. In twenty-one breast muscle operated birds on which weight changes were recorded the average weight loss was 2.33% (range +2% to −8%, all but one between +2% and −5%), the average ovulation frequency 0.62, and the incidence of atresia 9.5% (2 out of 21 birds). It can be seen from Figure 2 that an incidence of atresia of approximately 40%

TABLE 4. RELATION BETWEEN OVULATION FREQUENCY AND THE INCIDENCE OF ATRESIA IN THE M_1 FOLLICLE IN 205 ABDOMINALLY OPERATED BIRDS, WHICH DID *NOT* OVULATE ON THE DAY AFTER OPERATION. THE NUMBERS IN PARENTHESES ARE THE NUMBER OF BIRDS SHOWING ATRESIA IN EACH GROUP.

Ovulation frequency groups							
0.43–0.60		0.61–0.70		0.71–0.80		0.81–0.93	
No. birds	% showing atresia	No. birds	% showing atresia	No. birds	% showing atresia	No. birds	% showing atresia
62	66.1% (41)	71	47.9% (34)	61	41.0% (25)	11	18.2% (2)

would be expected in abdominally operated birds of similar ovulation frequency and weight loss.

C. Relation between the Incidence of Atresia and Ovulation Frequency in Birds which Did Not Ovulate on the Day after Operation.

In order to eliminate the possibility that the birds with high ovulation frequencies are essentially the same in reactivity to whatever induces atresia as the birds with low ovulation frequencies, but show a lower incidence of atresia because they are more likely to ovulate on the day after operation (thus escaping the influence of the atresia-inducing agent), a reexamination of the data was made with only those birds included which did *not* ovulate on the day after operation. The data are shown in Table 4. It can be seen that the inverse relationship between ovulation frequency and the incidence of atresia still holds.

D. Atresia in Maturing Follicles other than the M_1.

Of the 251 birds which were subjected to abdominal operations, 149 have been classified in the foregoing presentation as not showing atresia. The reasons for this classification have been set forth on page 419. Within this group of 149 birds there were 30 birds in which one or more of the other maturing follicles in the phase of rapid growth showed definite signs of atresia at autopsy, or if not autopsied, showed

by their lay records signs of having undergone atresia subsequent to operation.

For the sake of completing the picture so far presented, these birds have been included with those already classified as showing atresia, and the incidence of atresia compared with ovulation frequency in Table 5. It can be seen that although the incidence of atresia

TABLE 5. RELATION BETWEEN OVULATION FREQUENCY AND THE INCIDENCE OF ATRESIA IN THE M_1 OR IN ANY OF THE OTHER MATURING FOLLICLES IN THE RAPID GROWHT PHASE AFTER ABDOMINAL OPERATIONS. THE NUMBERS IN PARENTHESES ARE THE NUMBERS OF BIRDS SHOWING ATRESIA IN EACH GROUP.

Ovulation frequency groups							
0.43–0.60		0.61–0.70		0.71–0.80		0.81–0.93	
No. birds	% showing atresia	No. birds	% showing atresia	No. birds	% showing atresia	No. birds	% showing atresia
62	72.5% (45)	73	58.9% (43)	85	44.7% (38)	31	25.8% (8)

in each group is now higher, as expected, the inverse relation with the frequency of ovulation is still present.

DISCUSSION

We have attempted to demonstrate in the foregoing pages the fact that, other things being more or less equal, the most important single factor which determines whether or not a bird will undergo atresia of the maturing follicles following an operation (and, we may presume, following most other atresia-inducing agents which elicit a variable response)[2] is the frequency with which she has been ovulating before the operation. While this has been the primary purpose of this paper, it would not be out of place at this time to attempt an explanation of this relationship. It should, of course, be understood, that we have no experimental proof for this explanation.

Any attempted explanation of this relationship must assume a knowledge of the mechanism through which both "slow" and "fast" layers react to the given stimulus. In our view, the most probable mechanism would be one in which the pituitary is directly affected by the bodily changes induced by the operation. Some of the reasons for this postulation have been set forth below in the discussion of delayed ovulations.

On the basis of this assumed mechanism the observed relationship might depend on the fact that with increases in ovulation frequency,

[2] We wish to point out a distinction between such atresia-inducing agents as the above-described operations, injections of tissue or protein suspensions, etc., which elicit variable responses in relation to the size of the stimulus and the reactivity of the recipient, and such agents as hypophysectomy, which if complete, that is, above a required threshold, elicit an invariable response.

there is a concomitant increase in the level of the activity of the pituitary, or of the pituitary-ovary system, and that associated with this level of activity is the ability of the pituitary to withstand the atresia-inducing influences or agents resulting from the operations. The incidence of atresia is probably a direct measure of this resistance to change of the pituitary and it is evident from our data therefore that increases in this resistance must take place at a greater rate than the increases in ovulation frequency. That is, the drop in the incidence of atresia (i.e. increase in resistance) is of the order of 4 to 10 times the corresponding rise in ovulation frequency (Figure 2).

There is one possible relationship derived from these interpretations that deserves mention. First, it should be understood that the level of activity of the pituitary, which we have spoken of above, means primarily the level of basic activity of the gland (metabolic rate or rate of constant secretion of hormone or both) and is to be distinguished from the level of cyclic activity; and second, that the ovulation frequency is a direct measure of this cyclic activity. Following from these and the above considerations, it is very probable that increases in the value of the ovulation frequency reflect relatively much larger increases in the level of basic activity of the pituitary.

It should be pointed out that the above interpretations do not necessarily preclude the participation of estrogen or other strictly ovarian product in the manifestation of this relationship. The ovary undoubtedly contributes to the effectuation of a given response, but it is our belief that this contribution is of secondary importance in comparison with the part played by the pituitary itself.

Some evidence in favor of these interpretations if found in the incidence of delayed ovulations following the operations. (These have been mentioned above, and will be reported on in detail at a later date.) These delays in ovulations apparently have also been observed following injections of tissue and protein suspensions, etc., usually at levels less than those required to induce atresia (Fraps and Neher, 1945). In the series of birds operated on, the delays in ovulation occurred in birds showing relatively greater frequencies of ovulation than those of the birds undergoing atresia. The injected series were in general birds with similar and low frequencies of ovulation. Thus, the two series of experiments complement each other, the injected series of birds having a more or less uniform "resistance to change," but being subjected to stimuli of varying magnitude, the birds operated on having varying "resistance to change" but being subjected to a stimulus of approximately constant magnitude. A relatively small stimulus in the former series should therefore be equivalent to a relatively high resistance in the latter series. The fact that the first response after injection was delayed ovulation, the second atresia, and that atresia was more frequent in the birds with low ovulation frequencies (low

resistance), while delayed ovulations more frequent in these with higher ovulation frequencies (higher resistance) indicates that this is indeed so, and that both stimuli, therefore, probably induce atresia and delayed ovulations through the same mechanism, and that both atresia and delayed ovulations are qualitatively different responses of the same system.

The particular nature of the atresia-inducing changes induced in the organism by the operations is one of the most puzzling aspects of this whole phenomenon. Certainly the removal of an ovarian element is excluded from having a determining role by the fact that sham operations in which the ovary is not at all handled are followed by atresia to the same extent as after operations in which an ovarian element is removed. Injury effects and weight losses seem to be the same after both abdominal and breast muscle operations, yet the latter are almost ineffective in inducing atresia. Whatever the manner in which these operations act to induce atresia, the fact that they do so should make interpretation of past and future work relating to the specific effect of certain operative procedures on ovarian behavior more, or less difficult depending on the extent to which the effects of the operation per se are controlled.

Finally and incidentally, we would like to mention a point which further corroborates our previously reported finding that the removal of the most recently ruptured follicle leads to delay in the time of lay of the egg arising from it (Rothchild and Fraps, 1944). Of the 111 birds operated on for removal of the R_1 ruptured follicle, 103 were allowed to live beyond the time of expected normal lay. Of these, 82, or 79.6% showed delayed lays. Of 177 control operated birds, (140 of them subjected to abdominal operations) only one bird showed a delayed lay.

SUMMARY

The incidence of follicular atresia was studied in six groups of domestic hens subjected to one each of the following operations; removal of the most recently ruptured follicle (111 birds), removal of the next older follicle (51 birds), removal of a follicle older than either of the first two (39 birds), sham ovarian operation (35 birds), operation on the right side of the body cavity (sham) (15 birds), and operation on the breast muscle (37 birds). It was found that in all the groups which were subjected to abdominal operations the incidence of atresia was inversely proportional to the frequency of ovulation preceding operation. The induction of atresia was found to be associated with the effects of abdominal operation per se, rather than with the removal of an ovarian element. Operations performed on the breast muscle, however, resulted in a negligible incidence of atresia. It was also found that the incidence of atresia was directly related to the loss in body weight following operation, in birds of the same or similar ovula-

tion frequencies. However, proportionately much greater increases in the incidence of atresia occurred in birds of similar body weight changes in relation to decreases in ovulation frequency, than they did in birds of similar ovulation frequency in relation to increases in weight losses. A theoretical explanation of the relationship is offered, which is based on the assumption that the induction of atresia is mediated through the pituitary gland.

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THE SUCCINIC DEHYDROGENASE ACTIVITY OF OVARIAN AND LUTEIN TISSUE[1]

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As part of a program developed to study the relationship between hormones and intracellular enzymes, experiments were designed to determine the succinic dehydrogenase activity of the ovaries of immature rats which had been stimulated by gonadotrophic hormones, the ovaries of pregnant, pseudopregnant and of female rats having normal estrous cycles. As ovarian tissue in the rat is composed largely of follicles and corpora lutea, data were obtained concerning the amount of succinic dehydrogenase in the corpora lutea dissected from ovaries removed during the above phases of the reproductive cycle.

EXPERIMENTAL

Ovarian tissue was obtained in part from 26 day old immature rats which had been injected with follicle stimulating hormone (McShan and Meyer, 1940) and unfractionated preparations made from whole sheep pituitary powder (McShan and Meyer, 1943). The data obtained from these tissues were compared with those of immature rats not injected and of the same age. The rats used were of the Sprague-Dawley strain, obtained from Sprague-Dawley, Inc., or raised in our laboratory.

In addition to these tissues, studies were made of ovarian tissue removed from adult rats in diestrus, and from pseudopregnant and pregnant rats. The rats were made pseudopregnant by mechanical stimulation of the cervix during estrus and proestrus and the ovaries were removed on the 7th day of pseudopregnancy. The pregnant rats were killed at 18 to 20 days of gestation. The corpora lutea tissue was dissected from the other ovarian tissues of diestrous, pseudopregnant and pregnant rats. The activity of the residual ovarian tissue from pregnant rats was also determined.

The "homogenization" method of Potter and Elvehjem (1936) for studying tissue oxidations was used in making the tissue preparations which will be referred to as a "homogenate." The ovaries or lutein tissue were removed immediately after the rats were killed by decapitation, placed in a homogenizing tube with 0.5 ml. of glass distilled water, and homogenized immediately. Sufficient water was added to make a 5 per cent homogenate, and the proper amounts of this suspension were placed in Warburg flasks that contained cold fortified substrate.

The fortified substrate solution consisted of 1 ml. 0.1 M phosphate buffer

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of pH 7.4, 0.3 ml. 0.5 M sodium succinate, 0.2 ml. 3×10^{-4} M cytochrome c, 0.3 ml. each of 4×10^{-3} M $AlCl_3$ and $CaCl_2$ plus water to make a total volume of 3 ml. per flask (Potter, 1941; Potter and Schneider, 1942). The center cups contained 0.1 ml. of 20 per cent potassium hydroxide. Approximately 20 minutes were required from the time the tissue was removed from the animal until it was placed in the apparatus at 38° C. The contents of the flasks were allowed 10 minutes to come to equilibrium with the temperature of the water bath after which readings were made at 10 minute intervals for 1 hour. The results are reported in terms of Q_{O_2}, i.e., the oxygen uptake per milligram of dry tissue per hour. The dry weights expressed as percentage of wet weight of the different kinds of ovarian tissue are given in Tables 2 and 3. These values were obtained by drying ovarian and lutein tissues, and

TABLE 1. OXYGEN UPTAKE AS RELATED TO THE AMOUNT AND KIND OF OVARIAN TISSUE REACTING

Kind of ovaries	Amt. of tissue	Oxygen uptake per 10 minutes[1]
	ml. 5% homogenate	cmm.
Immature rats:	0.2	5.7
	0.3	8.9
Stimulated—only follicles	0.4	12.3
	0.5	15.8
Stimulated—follicles and corpora	0.3	10.0
	0.4	12.9
	0.5	16.1
Adult:		
Diestrous—follicles and corpora	0.2	6.7
	0.3	10.9
	0.4	13.6
Pregnant 18–20 days—follicles and large corpora	0.2	13.0
	0.3	20.2
	0.4	27.4
	0.5	33.3

[1] Average value for first four ten minute periods.

by drying aliquots of their homogenates at 75° C. for 24 hours. A 0.5 M solution of Eastman sodium succinate was brought to pH 7.4, and used as substrate.

The cytochrome c was made from beef heart muscle according to the method of Keilin and Hartree (1937) except that the final product was dialyzed against distilled water instead of 1 per cent sodium chloride solution as sodium chloride inhibits the succinoxidase system (Potter, 1941). The concentration of cytochrome c was determined by use of the Evelyn photolometer (Umbreit, Burris and Stauffer, 1945).

By the use of a constant amount of 5 per cent ovarian homogenate with varying mounts of 3×10^{-4} M cytochrome c it was found that 0.15 ml. was sufficient to elicit the maximum succinic dehydrogenase activity of this particular tissue. However, 0.2 ml. of cytochrome c was used in the experiments so as to be sure that the concentration was sufficiently great in all

cases. The data of Table 1 show that the succinic dehydrogenase activity of homogenates of ovaries from immature rats which were given gonadotrophins and ovaries obtained from normal adult and pregnant rats was directly proportional to the amount of homogenate used.

RESULTS AND DISCUSSION

The first experiments to be discussed were done for the purpose of determining what effect F.S.H. and unfractionated pituitary preparations would have on the succininc dehydrogenase activity of the ovaries of immature rats. The activity of unstimulated ovaries of immature rats was determined for comparative purposes. The data in Table 2 show that the succininc dehydrogenase activity is somewhat greater in the unstimulated ovaries than in those of animals receiving

TABLE 2. SUCCINIC DEHYDROGENASE ACTIVITY OF OVARIAN TISSUE OF THE RAT

Kind of ovaries	Dry weight-% of wet wt.	No. of runs	Q_{O_2}	
			Average	Range
Immature rats:				
Not stimulated	22.0	2	24.8	(20.4–28.5)
Stimulated—few to several corpora	16.7	6	19.6	(15.0–23.6)
Stimulated—numerous corpora[1]	16.7	3	20.6	(17.1–24.5)
Stimulated—only follicles[1]	14.8	3	20.8	(17.5–24.6)
Adult:				
Nonpregnant in diestrus—follicles and corpora	21.6	7	18.6	(15.0–24.0)
Pregnant 18–20 days—large corpora	22.1	6	32.6	(27.8–49.9)
Pregnant 20 days—large corpora	22.1	2	32.7	(30.4–36.4)
Pseudopregnant 7 days—corpora	21.2	3	27.5	(22.9–31.1)

[1] The rats were injected with gonadotrophin for 4.5 days and killed on the morning of the sixth day when the animals were 26 days old.

gonadotrophins. This result was not expected, but when the quantity of follicular fluid in the stimulated ovaries is considered it becomes apparent that it would be a greater diluting factor than the fluid in unstimulated ovaries, in which the follicles are small and contain relatively little, if any, follicular fluid. This explanation is supported by the dry weight determinations given in Table 2. Whether or not this explanation is correct, the fact remains that the succinic dehydrogenase content of stimulated ovaries containing both corpora lutea and follicles is less than that of unstimulated ovaries. The values for ovaries composed of follicles only are almost identical with the values for those containing few or numerous corpora lutea. This result was not predicted and it was decided to ascertain whether the succinic dehydrogenase content of ovaries removed from adult rats in diestrus was different or like that of ovaries of immature animals which had received gonadotrophins. The values obtained are to be found in Table 2 and they demonstrate that the dehydrogenase activity of ovaries

from animals in diestrus is less than that of immature rats whose ovaries contained both follicles and corpora lutea.

At this stage in the study the data just discussed provided the basis for suggesting the hypothesis that lutein tissue in the ovaries of rats in diestrus and immature rats injected with gonadotrophins is not highly functional, and that the succinic dehydrogenase activity might, therefore, be low. This hypothesis was tested in part by determining the succinic dehydrogenase activity of ovaries of pseudopregnant and pregnant rats in which there are corpora lutea which are highly functional. The results obtained (Table 2) show that the ovaries

TABLE 3. SUCCINIC DEHYDROGENASE ACTIVITY OF LUTEIN TISSUE FROM THE OVARIES OF PREGNANT AND PSEUDOPREGNANT RATS AND RATS IN DIESTRUS

Kind of rat	Kind of tissue	Dry weight % of wet wt.	No. of runs	Q_{O_2}	
				Average	Range
Diestrous	lutein	24.4	2	18.5	(16.0–19.9)
Pregnant	lutein	22.2	3	46.8	(41.2–54.2)
Pregnant	residual ovarian after corpora were removed	20.6	1	24.5	
Pseudopregnant 7 days	lutein	22.2	2	43.6	(42.2–44.2)

of rats in these phases of the reproductive cycle have a much greater succinic dehydrogenase activity as compared with that of ovaries of immature animals given gonadotrophins, or of adult females in diestrus.

It is possible, however, that the succinic dehydrogenase activity of the other tissues of the ovary as well as that of the corpora lutea is greater than those of immature rats, or of adult rats in diestrus. It seemed important, therefore, to study the dehydrogenase activity of lutein tissue dissected free from the other tissues of the ovaries of pseudopregnant and pregnant rats, and rats in diestrus. The data obtained show that corpora lutea from pseudopregnant and pregnant rats are high in succinic dehydrogenase activity when compared with that found in the corpora lutea of rats in diestrus (Table 3). The data also support the concept that the high dehydrogenase activity of the ovaries of pregnant and pseudopregnant rats is due to the functioning corpora lutea which they contain (Tables 2 and 3). This idea was tested further by determining the succinic dehydrogenase activity of the luetein tissue and residual ovarian tissue of pregnant rats. The results given in Table 3 show that the activity of the dehydrogenase in the ovarian residue is low as compared with that of lutein tissue.

There seems to be no reason to doubt that the corpora lutea of pregnant and pseudopregnant rats are functional and that the lutein tissue of adult rats in diestrus (Long and Evans, 1922) or that of im-

mature rats produced by gonadotrophins (Shelesnyak, 1931; Astwood, 1941) have the potentiality for a high degree of function if the appropriate hormonal factors act. As a high level of succinic dehydrogenase activity has been demonstrated for functioning lutein tissue of rats and a low level for corpora lutea having little if any function, it is apparent that there is a direct correlation between the succinic dehydrogenase activity and the high and low levels of lutein tissue function. On this basis it is suggested that the succinic dehydrogenase activity of corpora lutea could be used as a measure of function. This method would be of value in ascertaining whether the corpora lutea are functional in the ovaries of animals during certain phases of the reproductive cycle and under various experimental conditions where it is often difficult, if not impossible, to determine whether they are functional by the standard techniques now available. It also seems reasonable to believe that succinic dehydrogenase determinations could be used to ascertain the degree of function of a single corpus luteum, or of the corpora lutea of one kind where there is more than one kind present in the ovary at the same time.

The question of the relation of the pituitary gland to the succinic dehydrogenase activity and function of the corpus luteum is one of interest. It has been shown that the function of corpora lutea in the rat is dependent upon the luteotrophic activity of the lactogenic hormone of the pituitary gland (Astwood, 1941), and also that extracts of rat placentae will maintain luteal activity (Astwood and Greep, 1938). We have unpublished data which show that the succinic dehydrogenase of the corpora lutea of hypophysectomized rats given lactogenic hormone is equal to that of lutein tissue from pregnant rats. These data suggest that the energy needed for the processes involved in the secretion of progesterone may be derived from the oxidation of pyruvate to carbon dioxide and water by way of the Krebs cycle. It is of interest to note that the oxidation of succinate to fumarate is an essential link in this cycle by which energy is made available for cellular processes, and that succinic dehydrogenase catalyzes this oxidation (Krebs, 1943). It may well be that the lactogenic hormone exerts its effect in this process by directly or indirectly bringing about an increased activity or concentration of the energy mobilizing enzymes which are concerned with the oxidations of the Krebs cycle, and that the succinic dehydrogenase assay is a measure of the increased functioning of the cycle, which in turn is correlated with the increased function of the lutein tissue.

SUMMARY

The succinic dehydrogenase activity was determined in: 1) the ovaries of immature rats, immature rats given gonadotrophins, adult pregnant and pseudopregnant rats and rats in diestrus; 2) lutein tis-

sue only of pregnant, pseudopregnant and rats in diestrus; and 3) the other ovarian tissues remaining after removal of the corpora lutea from ovaries of pregnant rats.

The high succinic dehydrogenase activity of the ovaries of pregnant and pseudopregnant rats is due to the high activity of the functional lutein tissue. Conversely, the low succinic dehydrogenase activity of lutein tissue from the ovaries of rats in diestrus, or immature rats given gonadotrophins, probably accounts for the low activity of this enzyme in ovaries of these types. It is suggested that the succinic dehydrogenase activity of lutein tissue might be used as a criterion of the level of function of corpora lutea.

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THE INFLUENCE OF URINARY CORTIN-LIKE MATERIAL ON SODIUM AND POTASSIUM METABOLISM[1]

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IT HAS BEEN demonstrated that urinary extract contains material which possesses certain biological activities similar to those of the steroid hormones of the adrenal cortex. Extracts of normal men's urine showed the following biological activities when tested on the adrenalectomized rat:

1. Increased resistance to low environmental temperatures (Dorfman, Horwitt and Fish, 1942).
2. Maintained life (Dorfman and Horwitt, 1943).
3. Increased liver glycogen deposition in the fasting animal (Horwitt and Dorfman, 1943).
4. Prolonged the average work performed by the gastrocnemius muscle (Shipley, Dorfman and Horwitt, 1943).
5. Prevented water intoxication (Schiller and Dorfman, 1943).

It has also been reported that the urine of postoperative patients contains material which is biologically active in the adrenelectomized rat with respect to life maintenance, resistance to low environmental temperatures, and glycogen deposition in the liver of the fasted animal (Weil and Browne, 1939a and 1939b; Venning, Hoffman and Browne, 1943).

This report is concerned with studies on the influence of urinary cortin-like material on sodium and potassium metabolism. The studies on sodium were done with normal fasted rats. The influence on potassium metabolism was studied by means of a potassium intoxication test in the adrenalectomized rat.

METHODS, ANIMALS AND MATERIALS

Sodium Excretion Test.

Male albino rats weighing between 80 and 200 grams, after a twenty-four hour fast, received 2 cc. of 10 per cent ethanol or an extract dissolved in 2 cc. of 10 per cent ethanol by stomach tube. One half hour later the rats

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received 0.5 cc. of a 10 per cent sodium chloride solution. Each rat was placed in an individual metabolism cage and the urine collected under toluene. During the experimental period, usually four hours, the rats received distilled water but no food. At the end of the experimental period the cages were rinsed with distilled water. The sodium content in the urine was determined by precipitation of sodium as uranyl zinc sodium acetate (Peters and Van Slyke, 1932). Duplicates were run on each sample.

Potassium Intoxication Method.

The procedure for studying the effect of urinary extract, adrenal cortical extract, and desoxycorticosterone on potassium metabolism is a modification of the potassium intoxication test of Truszkowski and Duszynska (1940) used in adrenalectomized mice. Male albino rats were bilaterally adrenalectomized by the dorsal route under ether anesthesia. The extract or hormone in 10 per cent ethanol was administered by stomach tube in divided doses before the potassium chloride solution was injected. The potassium chloride (a 3.3 per cent solution) was administered intraperitoneally on the basis of body weight. During the test, each rat was placed in an individual jar without food or water, and was observed for one to two hours. The control rats received a total of 1.5 or 3.0 cc. of 10 per cent ethanol, a volume equivalent to that administered to the extract treated rats.

Animals.

The rats employed in these studies were of the Sprague-Dawley and Rockland Farm strains. Immature and young adult male rats were used for the various studies. The animals were purchased from Sprague-Dawley, Madison, Wisconsin, and Rockland Farms, New York, and kept in the laboratory for varying lengths of time before using. While in the laboratory they were fed Purina Fox Chow without supplements.

Materials.

Commercial extracts of the adrenal cortex prepared by the Wilson Laboratories and the Upjohn Company were used. The Upjohn extract was employed for the sodium excretion studies since it contained no sodium chloride. Desoxycorticosterone was administered in the form of its acetate.[2]

The urinary extract was prepared by the extraction of normal men's urine with chloroform or ethylene dichloride at room temperature. Two fractions of the urinary extract, the alkali insoluble and the alkali insoluble ketonic fractions were employed. The extracts and desoxycorticosterone acetate were suspended in 10 per cent ethanol and administered by stomach tube. The volumes varied from 0.5 to 1.0 cc. of solution for each administration. Extract treated rats were run simultaneously with control rats which received an equal volume of the solvent, 10 per cent ethanol.

EXPERIMENTAL

Sodium Excretion Studies

After the administration of 51.1 mg. of sodium equivalent, 1.3 cc. of 10 per cent sodium chloride solution, to six fasted male rats

[2] We are indebted to Ciba Pharmaceutical Products, Inc. for the supply of Desoxycorticosterone Acetate.

weighing 77 to 86 grams, the excretion of sodium in the urine was determined in three successive four hour periods (Table I). The greatest amount of sodium, 26.7 to 42.8 mg. (mean of 36.2 mg.) was excreted in the first four hours. The excretion of sodium decreased in the next four hours when 5.2 to 17.3 mg. (mean of 10.3 mg.) of sodium were recovered. There was a further decrease, 1.6 to 3.6 mg. (mean of 2.9 mg.) in the last four hour period. At the end of twelve hours, 46.3 to 51.7 mg. (mean of 49.4 mg.) of the 51.1 mg. of sodium that had been administered were recovered. Since the percentage of total sodium excreted decreased in the successive four hour periods, 73.3, 20.8, 5.9, and the largest amount of sodium was excreted in the first period, the first four hour period after sodium administration was used to determine the effect of different extracts on sodium excretion in fasted male rats.

TABLE I. THE EXCRETION OF SODIUM AFTER THE ADMINISTRATION OF SODIUM CHLORIDE TO FASTED MALE RATS

(Sodium Chloride Administered at Zero Time)

Wt. of rats after 24 hour fast	Amount of sodium chloride administered	Sodium excreted in successive 4 hour periods			Total sodium excreted in 12 hours
Gm.	Mg. of sodium	Mg.			Mg.
		1st	2nd	3rd	
86	51.1	37.5	9.8	3.3	50.6
79	51.1	40.6	9.5	1.6	51.7
80	51.1	26.7	17.3	2.3	46.3
80	51.1	42.8	5.5	3.6	51.9
86	51.1	38.0	5.2	3.3	46.5
77	51.1	31.5	14.4	3.5	49.4
	Mean =	36.2	10.3	2.9	49.4
Per Cent of Total Excretion		73.3	20.8	5.9	—

During the twelve hours of urine collection a total of 97 per cent of the administered sodium was recovered. Of the total amount of sodium excreted during the twelve hours the first four hour collection accounted for 73.3 per cent while the second and third four hour periods amounted to 20.8 and 5.9 per cent respectively.

The influence of Upjohn Adrenal Cortical Extract (Table II) on the excretion of sodium was determined after the administration of sodium chloride to the fasting male rat. Nine rats weighing 98 to 202 grams received 2 cc. of 10 per cent alcohol one-half hour before the administration of 19.7 mg. equivalent of sodium which was contained in 0.5 cc. of a 10 per cent sodium chloride solution. The sodium excreted in the first four hours varied from 3.5 to 13.9 mg. The mean was 9.9 mg. The ten rats which received 2 cc. of adrenal cortical extract

one-half hour before the administration of 19.7 mg. of sodium, excreted 13.4 to 29.9 mg. of sodium in the first four hours. The mean was 22.1 mg. of sodium. The increase in sodium excretion caused by the adrenal cortical extract was 123 per cent and was found to be statistically significant (Fischer, 1932).

Adrenal cortical extract produced an increased excretion of sodium

TABLE II. THE INFLUENCE OF ADRENAL CORTICAL EXTRACT ON THE EXCRETION OF SODIUM AFTER THE ADMINISTRATION OF SODIUM CHLORIDE TO FASTING MALE RATS

Experiment	Sodium chloride administered ½ hour after extract	Adrenal cortical extract administered	Number of rats	Mean wt. of rats after 24 hour fast	Mean sodium excreted in urine in 1st 4 hours	Change in sodium excretion in %
	Mg. of Na.	c.c		Gm.	Mg.	
A	19.7	0	9	149 (98–202)	9.9 (3.5–13.9)	
	19.7	2	10	137 (86–187)	22.1 (13.4–29)	+123
B	39.4	0	3	100 (96–104)	31 (31–31)	
	39.4	2	3	102 (98–104)	43 (39–48)	+38.7

TABLE III. THE INFLUENCE OF DESOXYCORTICOSTERONE ACETATE ON THE EXCRETION OF SODIUM AFTER THE ADMINISTRATION OF SODIUM CHLORIDE TO FASTING MALE RATS

(Sodium chloride, 19.7 mg. of sodium equivalent, was administered to each rat ½ hour after the hormone)

Desoxycorticosterone acetate administered Mg.	Number of rats	Mean wt. of rats after 24 hour fast	Mean sodium excreted in urine in 1st 4 hours	Change in sodium excretion
0	11	118 (98–164)	14.6 (7.6–19.1)	
8	12	118 (102–155)	11.6 (6.9–18.2)	−20.5

when 39.4 mg. of sodium as sodium chloride was administered. Three fasting male rats weighing 96 to 104 grams that received 2 cc. of 10 per cent alcohol and 39.4 mg. of sodium, excreted 31 mg. of sodium during the first four hours. Three rats that received 2 cc. of adrenal cortical extract and 39.4 mg. of sodium excreted a mean of 43 mg.

Table III illustrates the influence of 8 mg. of desoxycorticosterone acetate on sodium excretion. In this experiment a definite retention of sodium is indicated. Eleven rats which only received the solvent

excreted between 7.6 and 19.1 mg. of sodium (mean of 14.6 mg.), during the first four hours. Twelve male fasting rats run simultaneously excreted 6.9 to 18.2 mg. of sodium, mean 11.6 mg. Thus a statistically significant retention of 20.5 per cent was found.

The influence of urinary extract on the excretion of sodium is represented in Table IV. When 2 liters equivalent of urinary extract were administered no significant change in sodium excretion was apparent. At dose levels of 4.5 liters and 9 liters of urine respectively increased excretions of sodium of 15.1 and 16.7 per cent were found. When the dose was still further increased to 14 liters equivalent of urine an apparent retention was observed, but at the same time toxic symptoms were also observed. Alkali insoluble fractions were employed for these sodium excretion studies.

TABLE IV. THE INFLUENCE OF URINARY EXTRACT ON THE EXCRETION OF SODIUM AFTER THE ADMINISTRATION OF SODIUM CHLORIDE TO FASTING MALE RATS
(Sodium chloride, 19.7 mg. of sodium equivalent, was administered to each rat ½ hour after the extract)

Experiment	Urinary extract equivalent per liter administered	Number of rats	Mean wt. of rats after 24 hour fast	Mean sodium excreted in urine in 1st 4 hours	Change in sodium excretion
			Gm.	Mg.	%
F	0	3	106 (100–110)	15.6 (13.2–17.0)	
	2.0	3	107 (98–113)	15.9 (11.6–19.2)	+2
G	0	12	95 (82–106)	11.9 (4.4–16.4)	
	4.5	11	93 (80–110)	13.7 (8.2–22.6)	+15.1
H	0	5	97 (94–116)	13.8 (11.6–16.5)	
	9	5	104 (95–109)	16.1 (10.1–22.7)	+16.7
I	0	3	89 (86–96)	11.0 (4.7–19.1)	
	14	3	90 (86–92)	9.1 (4.6–13.0)	−17.3

Potassium Intoxication Studies

Under proper conditions of adequate amounts of glandular extract, desoxycorticosterone acetate or urinary extract, proper time of administration of these materials and optimal concentration of potas-

TABLE V. THE INFLUENCE OF ADRENAL CORTICAL EXTRACT ON THE POTASSIUM INTOXICATION OF THE ADRENALECTOMIZED MALE RAT

Time between adrenalectomy and potassium administration	Time between last administration of extract or solvent and potassium administration	Amount of potassium chloride administered	10% ethanol treated rats			Adrenal cortical extract treated rats				
Days	Hours	Mg/Gm. body weight	Number of rats	Mean body weight Gm.	Per cent dead	Number of rats	Mean body weight Gm.	Total amount of A.C.E. administered Cc.	Time between operation and administration Hours	Per cent dead
2	0.5	0.45	14	42	57	10	42	0.15	48; 51; 52.5	0
2	1.0	0.50	10	43	100	10	42	0.30	48; 51; 52.5	30
2	1.0	0.45	16	44	100	10	43	0.30	48; 51; 52.5	0
2	0.5	0.35	12[1]	44	75	12	46	0.60[2]	48; 51; 52.5	0
2	0.5	0.45	14	42	57	10	43	0.60	48; 51; 52.5	0

[1] These rats received 3 cc. of 0.9% saline.
[2] Wilson Extract was used.

sium chloride it was possible to demonstrate the protective effect of these materials. In all tables on potassium intoxication, the experimental animals are listed alongside the controls run simultaneously. These controls were treated in the same way as the experimental animals except that the control animals received only the solvent.

Table V describes a series of experiments in which 2 days elapsed between bilateral adrenalectomy and the potassium chloride administration. A total dose as low as 0.15 cc. of adrenal cortical extract proved to protect the operated rats against the toxic effects of the potassium. In the first experiment 57 per cent of 14 control rats died while none of the 10 experimental animals were dead at the end of the experimental period. Similar results were found in four more experiments, where the total dose of adrenal cortical extract was increased to 0.3 cc. and 0.6 cc. Two experiments at the dose level of 0.3 cc. showed for the controls 100 per cent deaths for both experiments while the adrenal cortical extract treated rats showed 30 and 0 per cent deaths respectively. When the dose level was increased to 0.6 cc. the deaths in the control groups were 75 and 57 per cent respectively as compared to no deaths in either of the experimental groups.

Table VI represents a series of 6 experiments done under standardized conditions with respect to all variables except dosage of desoxycorticosterone acetate, except in one instance where 0.5 mg. per gram of body weight of potassium chloride was administered instead of 0.45 mg. At a total dose of 0.75 mg. of desoxycorticosterone acetate in 2 experiments a definite protection was found in one. Thus in the first experiment 100 per cent of 10 controls died while 70 per cent of 10 treated animals died; in the second experiment 57 per cent of 14 controls died during a time when none of 8 experimental animals died. When the dosage was increased to 1.5 mg. total dose, good protection was found in each of 2 experiments. The comparative percentage deaths were 100 and 57 for the controls as compared to 50 and 20 respectively for the desoxycorticosterone acetate treated animals. At 2.25 and 3.0 mg. of hormone respectively, the controls were 57 and 57 per cent. While the corresponding percentages of 20 and 33 per cent were found for the hormone treated animals.

Of the total of 8 experiments run 2 days after bilateral adrenalectomy (Table VII) all showed protection at urine extract doses ranging from a total of 3 to 6 liters equivalent. The dosage of potassium chloride varied from 0.20 mg. per gram of body weight to 0.50 mg. per gram of body weight. At the level of three liters of urine the control deaths were 30 and 81 per cent respectively as compared to 0 and 22 per cent for the urinary extract treated animals. The six experiments at the 6.0 liter dose level all showed protection. If one considers all of the observations at the 6.0 liter level together, it is seen that 69 per cent of 83 ethanol treated rats died while only 11 per cent of the 55 rats which received the urinary extract died.

TABLE VI. THE INFLUENCE OF DESOXYCORTICOSTERONE ACETATE ON THE POTASSIUM INTOXICATION OF THE ADRENALECTOMIZED MALE RAT

Time between adrenalectomy and potassium administration	Time between last administration of extract or solvent and potassium administration	Amount of potassium potassium chloride administered	10% ethanol treated rats			Desoxycorticosterone treated rats				
Days	Hours	Mg/Gm. body weight	Number of rats	Mean body weight Gm.	Per cent dead	Number of rats	Mean body weight Gm.	Total amount of D.C.A. administered Mg.	Time between operation and administration Hours	Per cent dead
2	0.5	0.50	10	43	100	10	47	0.75	48; 51; 52.5	70
2	0.5	0.45	14	42	57	8	42	0.75	48; 51; 52.5	0
2	0.5	0.45	16	44	100	10	46	1.50	48; 51; 52.5	50
2	0.5	0.45	14	42	57	10	38	1.50	48; 51; 52.5	20
2	0.5	0.45	14	42	57	10	45	2.25	48; 51 52.5	20
2	0.5	0.45	14	42	57	9	47	3.00	48; 51; 52.5	33

TABLE VII. THE INFLUENCE OF URINARY EXTRACT ON THE POTASSIUM INTOXICATION OF THE ADRENALECTOMIZED MALE RAT

Time between adrenalectomy and potassium administration	Time between last administration of extract or solvent and potassium administration	Amount of potassium chloride administered	10% ethanol treated rats			Urinary extract treated rats				
Days	Hours	Mg/Gm. body weight	Number of rats	Mean body weight Gm.	Per cent dead	Number of rats	Mean body weight Gm.	Total amount of urinary extract administered L.	Time between operation and administration Hours	Per cent dead
2	0.5	0.20	10	52	30	9	48	3.0[1]	48; 51; 52.5	0
2	0.5	0.30	16	46	81	9	46	3.0[2]	48; 51; 52.5	22
2	0.5	0.40	17	45	41	13	48	6.0	48; 51; 52.5	0
2	0.5	0.20	10	52	30	10	47	6.0[1]	48; 51; 52.5	0
2	0.5	0.30	16	46	81	10	43	6.0[2]	48; 51; 52.5	10
2	0.5	0.30	14	47	57	3	46	6.0[2]	48; 51; 52.5	0
2	1.0	0.50	10	43	100	10	45	6.0[3]	48; 51; 52.5	30
2	1.0	0.45	16	44	100	9	46	6.0[3]	48; 51; 52.5	22

[1] The alkali insoluble fraction of a chloroform extract was used.
[2] The ketonic fraction of the chloroform extract was used.
[3] The alkali insoluble fraction of an ethylene dichloride extract was used.

DISCUSSION AND CONCLUSIONS

The studies reported here indicate that the urinary extracts of normal men may cause increased sodium excretion in a manner similar to that found for adrenal cortical extract and secondly that these extracts can protect against potassium intoxication.

The experimental conditions of the sodium excretion test have been narrowed to rather fine limits. The emphasis has been placed on short time excretion studies in the fasting normal rat which received the test material one half hour before the standard dose of sodium chloride was administered. The urine was collected for only four hours from the time of the salt administration. The individual rats did not serve as their own controls. However, the control rats receiving only the solvent were run simultaneously. The comparisons of the activities of adrenal cortical extract, desoxycorticosterone acetate and urinary extract are based solely on these specific conditions.

The standard dose of 19.7 mg. of sodium equivalent seemed convenient, since during the first four hours an adequate amount of sodium was excreted to permit the analysis either under conditions of no treatment or under conditions of increased excretion or retention. The untreated animals excreted about 50 to 75 per cent of the administered sodium within the first four hours after the administration of the sodium chloride. When a larger amount of sodium chloride was administered about the same magnitude of sodium equivalent was excreted. At the level of 51.1 mg. equivalent of sodium, a mean of 36.2 mg. of sodium was found for the first four hours excretion or 71 per cent of the administered sodium.

Under the conditions of the sodium experiments, adrenal cortical extract produced an increment in sodium excretion rather than a retention. When 2 cc. of extract were used and 19.7 mg. of sodium were administered, the increase in sodium excretion was 123 per cent. At a higher level of sodium administration, 39.4 mg. equivalent of sodium, 2 cc. of adrenal cortical extract caused a 38.7 per cent increase in sodium excretion. Thorn *et al.* (1937) found an increased sodium excretion in normal humans during a twenty-four hour period after cortin administration. He believed that the effect was due to atmospheric conditions since normal humans, who received no treatment, showed increased excretion of sodium at the same time. When Thorn increased the dose of extract he found sodium retention in normal humans. In the experiments of West (1942), normal rats of different stocks were kept on a standard diet, and twenty-four hour sodium excretion studies were done on groups of 4 rats. His results were inconsistent. Some rats retained sodium, others excreted it. Harrop and Thorn (1937) found that sodium retention occurred in four hour excretion studies of normal dogs that had received adrenal cortical extract.

The reason for the increased excretion of sodium rather than decreased excretion after the administration of adrenal cortical extract is not apparent at this time. One possible explanation involves the question of species differences. Certain of the sodium excreting steroids such as 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone present in the extract (Thorn, 1941) may cause a relatively greater effect in the rat under the conditions of our experiments than has been found previously in dogs and humans. Secondly, the sodium retaining factors such as desoxycorticosterone, may exert a relatively weaker action. Indications that this later factor may be real comes from the fact that under the experimental conditions employed 8 mg. of desoxycorticosterone acetate were necessary to produce retention of sodium.

In the experiments which concerned the influence of alkali insoluble urinary extract on the excretion of sodium, two liters of urinary extract had no effect. The levels of 4.5 and 9.0 liters tended to show an increased excretion of 15 per cent. But the increased excretion was not statistically significant on the basis of the number of experiments that were run. The toxicity of 14 liters prevented the further use of larger doses. However, more conclusive results might be obtained if the urinary extract were purified by separation into its ketonic fraction and larger doses of this latter fraction were administered.

It has been demonstrated that urinary extracts, both alkali insoluble fractions and alkali insoluble ketonic fractions, contain material which has an influence on potassium metabolism in a manner similar to that found for both adrenal cortical extracts and desoxycorticosterone acetate. Thus adrenalectomized rats were protected from the toxic effects of administered potassium by the urinary extracts employed.

Truszkowski and Duszynska (1940) were able to show that desoxycorticosterone acetate, but not adrenal cortical extract, protected adrenalectomized mice from toxic doses of potassium. Under their experimental conditions, immature male Albino mice weighing 8.5 to 12.5 grams were adrenalectomized one hour after a subcutaneous injection of 0.1 to 0.4 cc. of adrenal cortical extract. A similar dose was given on the two following mornings. After the last hormone injection 0.6 mg. of potassium chloride per gram of body weight was injected intraperitoneally within three hours. Since the mortality rate forty-eight hours after operation was an average of 71.5 per cent for extract treated mice and 73 per cent for untreated adrenalectomized mice, the adrenal cortical extract gave no protection against potassium intoxication under the given conditions. The failure of the adrenal cortical extract to protect the adrenalectomized mice from potassium intoxication may be a result of a low concentration of

extract or of the species of animal used. When Truszkowski and Duszynska injected a total of 1.5 mg. of desoxycorticosterone acetate within three days after adrenalectomy, the mice resisted 0.6 mg. of potassium chloride per gram of body weight injected forty-eight hours after operation.

It is interesting to note that Emmens and Marks (1942), while confirming the work of Truszkowski and Duszynska (1940) protected adrenalectomized mice with 1.0 and 2.0 mg. of sodium chloride per gram of body weight against 0.9 mg. of potassium chloride per gram of body weight. They were unable to protect adrenalectomized mice with 1 to 2 mg. of desoxycorticosterone acetate against 0.8 mg. of potassium chloride per gram of body weight.

By comparison of the present experiments with those of the foregoing authors, adrenalectomized rats seem to be more sensitive on the basis of body weight than adrenalectomized mice to injected potassium. It seems unlikely in this work that sodium chloride which can protect adrenalectomized mice was present in either the adrenal cortical extract or the desoxycorticosterone acetate, since both were 10 per cent ethanol solutions. The urinary extract was also free from water soluble salts and was diluted with 10 per cent ethanol.

In the course of the work it was found that young male adrenalectomized rats were suitable for the test. An interval of two days between bilateral adrenalectomy and the administration of the potassium salt seemed to be optimum. At one day after operation the difficulty found in protecting the operated rats was probably a result of the recent trauma of operation. In addition to this factor, it was found that a rather delicate balance existed between a number of other factors, namely: (a) time of administration of potassium chloride and of the administration of the test solution; (b) concentration of potassium chloride administered; (c) quantity of active protecting material administered. The most striking protections were demonstrated when the biologically active material was administered within 4 to 6 hours of the time of potassium chloride administration. A factor which must be emphasized is the amount of potassium chloride employed. The correct dose must be ascertained at the time of running each experiment. It was necessary to find the correct dose by trial and error before each experimental run. Doses of as little as 0.15 cc. of adrenal cortical extract were found to give significant protection against potassium intoxication; 0.75 mg. of desoxycorticosterone acetate was active; and extracts equivalent to as little as 1 liter of urine showed definite protection.

SUMMARY

It has been demonstrated by a potassium intoxication test in adrenalectomized rats that urinary extracts, both alkali insoluble and alkali insoluble ketonic fractions, contain material which has an

influence on potassium metabolism comparable to that exhibited by both adrenal cortical extracts and desoxycorticosterone acetate. Under the specific experimental conditions employed for studying sodium excretion in normal rats, it has been demonstrated that adrenal cortical extract caused a definite increase in sodium excretion, that desoxycorticosterone acetate caused a significant retention of sodium, and that the alkali insoluble urinary extract tended to cause an increase in sodium excretion similar to the effect of the adrenal cortical extract.

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RELATION OF CARBOHYDRATE DEFICIENT DIETS TO THE EFFECTIVENESS OF THE HORMONES OF THE ADRENAL CORTEX[1]

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IT IS WELL KNOWN that variations in the electrolyte content of the diet affect the response of adrenalectomized animals to cortical hormones. The effect of variation in other dietary constituents has been studied to some degree, but less extensively (Long 1942, Eversole 1945a). It is generally agreed that the adrenal steroids typified by desoxycorticosterone affect particularly electrolyte metabolism, whereas the 11-oxygenated steroids have a primary or at least potent action on carbohydrate metabolism, one phase of which is generally considered to be the stimulation of gluconeogenesis (literature cited by Swingle and Remington, 1944). Most workers have found that the deficiencies in carbohydrate metabolism seen in adrenalectomized animals were relieved little if any by desoxycorticosterone whereas the 11-oxygenated steroids were highly active (Long, *et al.*, 1940; Kendall, 1942; Ingle, 1942).

The present investigations were undertaken to see if desoxycorticosterone could be effective at all in the chronic maintenance of adrenalectomized animals on a diet in which carbohydrate was either low or lacking. Its effects in this regard were compared with those of a whole extract of the adrenal cortex.

METHODS

Two diets, one deficient in carbohydrates and the other carbohydrate-free, were used in the experiments. The carbohydrate-deficient diet was prepared by and purchased from the General Biological Supply House and contained the following ingredients:

	Parts
Casein	35
Cellu-flour	55
Butter	5
Sodium chloride	1
Calcium carbonate	2
Yeast	2
Cod liver oil	40 drops per 1,000 gm. of diet.

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The carbohydrate-free diet was prepared in our laboratory and was a modification of a diet used previously (Eversole, 1945b) which was recommended by R. J. Williams[2] of the University of Texas. This diet, unlike the one used previously, contained 3 gm. of salt, per 1,000 gm. of diet and casein was substituted for dextrose making a total of 923 gm. of vitamin-test casein per 1,000 gm. of diet. Thus the mixture was extremely rich in protein and free of carbohydrates.

Young male rats were used in all experiments; they were fed a stock diet until reaching a weight of approximately 55 gm. and then placed on one of the two carbohydrate-deficient diets for a period of either 10 or 20 days, or until death. The majority were adrenalectomized and treated with either desoxycorticosterone acetate (DCA) or whole extract of the adrenal cortex (Wilson: 1 cc. derived from 75 gm. of adrenal tissue). Usually 10 rats were used for each type of experiment and both intact and adrenalectomized controls were run for each diet.

Records were kept until death of the animals or for 30 days in a few cases where animals survived indefinitely. Animals which died within 24 hours after the operation or those which lived for 30 days after treatment was discontinued were not averaged in with the others because such animals did not show changes characteristic of adrenal insufficiency.

DCA in oil was injected subcutaneously in daily doses ranging from 0.2 mg. to 1.5 mg. Furthermore, for each diet, a number of rats were injected daily with DCA in suspension prepared as follows: 60 mg. of crystals were dissolved in 12 cc. of absolute alcohol, this was diluted with 48 cc. of water making a flocculated suspension of DCA in 20% alcohol. This was injected in 0.5 cc. (0.5 mg. DCA) amounts daily.

Extract of the adrenal cortex was given in daily doses of 0.5 cc. in 3 different ways: 1) by single subcutaneous injections daily, 2) by 3 injections daily—0.2 cc. at 9 a.m., 0.1 cc. at noon and 0.2 cc. at 6 p.m., and 3) by mouth in the drinking water. In the latter case the daily intake of each animal was estimated and extract added to the water in such amounts that 0.5 cc. was consumed daily with only minor variations.

RESULTS

Stock diet.

In a previous report (Eversole, 1945b) it was shown that 0.2 mg. of DCA daily was highly effective in adrenalectomized rats fed our stock diet; 90% of the animals survived and showed a weight gain of approximately 4 gm. per day. For this study experiments were extended to the effectiveness of cortical extract on the stock diet. When 0.5 cc. of extract was injected subcutaneously daily, 90% of the adrenalectomized animals survived the 10 day experimental period and showed an average daily weight gain of 2.2 gm. Therefore, the hormone preparations used in these experiments were capable of maintaining adrenalectomized rats in good condition.

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[2] In the previous publication Dr. Williams was erroneously referred to as R. J. Harrison.

Carbohydrate-deficient diet (Table 1).

Ten rats for each type of experiment were given this diet for a 10 day period. Intact controls were maintained in good health and showed an average daily weight gain of 2.25 gm. Eight of 10 adrenalectomized controls died during the experimental period showing a slight average loss in weight. DCA in daily doses ranging from 0.2 mg. to 0.5 mg. and given in oil was of little benefit; the great majority of the animals died within ten days although most of the rats showed

TABLE 1. AVERAGE FIGURES SHOWING RESPONSE OF RATS ON THE CARBOHYDRATE-DEFICIENT DIET TO DCA AND ADRENAL CORTEX EXTRACT (CE) GIVEN DAILY FOR 10 DAYS

Treatment	Number animals	No. surviving 10 day period	Wgt. change gm./day	Survival days	No. rats discarded
Intact control	10	10	+2.25	Indefinite	0
Adrex. control	10	2	−0.25	6.3	1 (lost)
Adrex. 0.2 mg. DCA in oil daily	10	0	+0.36	4.3	0
Adrex. 0.5 mg. DCA in oil daily	9	2	+1.36	8.3	1 (adrenal tissue)
Adrex. 0.5 mg. DCA in suspension daily	10	3	+1.43	10.7	0
Adrex. 0.5 cc. CE injected once daily	10	4	−0.47	10.0	2 (died 24 hrs.)
Adrex. 0.5 cc. CE injected daily in 3 doses	10	8	−0.03	11.9	0
Adrex. 0.5 cc. CE daily in drinking water	9	9	+0.48	16.4	1 (adrenal tissue)
*Adrex. 0.5 cc. CE daily in drinking water	10	0	−1.28	6.2	0

* Different batch of food.

a slight gain in weight. DCA in suspension may have been slightly more beneficial, but still most of the animals died.

In the experiments where extract of the cortex was used the response was better, although somewhat erratic. In most cases there was little change in weight. Animals receiving only one injection per day gave the poorest results but in most cases when the extract was given in divided doses or in the drinking water the animals were markedly benefited as shown by survival of 17 of 19 animals in two groups of experiments.

However, in another group of ten rats there were no survivors when the extract was given in drinking water and the animals showed weight losses. We suspected that this result may have been due to variations in this lot of diet. Accordingly, therefore, a synthetic diet, free of carbohydrates and well fortified with salts and known vitamins, was prepared in our laboratory and used in the following experiments.

Carbohydrate-free diet (Table 2).

Intact control animals were maintained in good health on this diet for periods up to 20 days. Ten rats showed an average daily weight gain of 2.1 gm. per day. This is a substantial weight gain for rats deprived of exogenous carbohydrates and is indicative of a satisfactory carbohydrate-free diet. None of the adrenalectomized control rats survived the experimental period; the average survival time was 3 days and the weight loss was 2.7 gm. per day. DCA injected in 0.2 mg. doses daily was of little benefit but with 0.5 mg. 50% of the

TABLE 2. AVERAGE FIGURES SHOWING RESPONSE OF RATS ON THE CARBOHYDRATE-FREE DIET TO DCA AND ADRENAL CORTEX EXTRACT (CE) GIVEN DAILY FOR 10 DAYS

Treatment	Number animals	No. survived 10 day period	Wt. change gm. per per day	Survival days	No. rats discarded
Intact control	10	10	+2.10	Indefinite	0
Adrex. control	10	0	−2.70	3.0	0
Adrex. 0.2 mg. DCA in oil daily	10	1	−0.45	4.8	0
Adrex. 0.5 mg. DCA in oil daily	10	5	−0.11	10.7	1 (died 24 hrs.)
Adrex. 1–1.5 mg. DCA in oil daily	10	3	−0.22	7.8	0
Adrex. 0.5 mg. DCA daily in suspension	15	9	+0.50	Survivors treated for 20 days	2 (adrenal tissue)
Adrex. 0.5 cc. CE injected daily in one dose	10	1	−1.52	6.1	0
Adrex. 0.5 cc. CE injected daily in three doses	20	12	−0.58	9.6	0
Adrex. 5 cc. CE daily in drinking water	10	8	−0.72	10.1	0
Adrex. 0.5 cc. CE daily in drinking water	10	10	+0.46	treated for 20 days	1 (lived indefinitely)

animals survived. It seemed likely that higher doses in oil might prove even more beneficial but such was not the case when 1.0 to 1.5 mg. per day were injected—the response could not be improved with higher doses given in this manner. However, DCA was somewhat beneficial when injected in suspension as 60% of the animals survived a 20 day experimental period. This may have been due to more efficient absorption of the hormone administered in this way.

Animals benefited but little, if any, when 0.5 cc. of cortical extract was injected once daily, but when this same volume was given in divided doses or in drinking water the beneficial effects on survival were striking. The best results were obtained when the rats received the hormone in drinking water. It is probable that administration by this method was more effective because the extract was being consumed in small amounts almost continuously, thus simulating natural secretion from intact glands.

For those animals that survived a 10 day experimental period in

good condition the treatment was continued for 20 days. One group of ten rats given cortical extract survived the 20 days and showed a slight weight gain. The nine animals treated with DCA suspension also survived and showed an average weight gain of 1.3 gm. per day.

DISCUSSION

It is clearly indicated by these experiments that DCA in doses which are highly effective in maintaining adrenalectomized rats on an ordinary stock diet (Eversole 1945b) are almost but not entirely ineffective when carbohydrate is largely or wholly eliminated from the diet. Cortical extract is also less effective on carbohydrate-deficient diets than on balanced ones, but its efficacy is much less impaired by lack of carbohydrate than is that of DCA.

Obviously, the use of carbohydrate-free diets puts under strain the mechanism of endogenous carbohydrate production. The experiments reported here are consistent with much evidence that the 11-oxygenated adrenal steroids (present in cortical extract) are concerned with gluconeogenesis whereas DCA is not (references cited by Long, 1942; Ingle, 1942; Swingle and Remington, 1944). Swingle and Remington (1944) pointed out that some investigators believe that the action of cortical hormone may be more concerned with the catabolism of protein than in actual utilization of carbohydrate and Long (1942) has shown that corticosterones accelerate protein catabolism particularly when the organism is forced to form glucose from protein. It may well be that whole extract and corticosterones augment the conversion of fed protein to carbohydrate, thus explaining the beneficial results obtained in the experiments reported here where whole extract was used.

It is not surprising that DCA was somewhat effective in maintaining adrenalectomized rats fed a carbohydrate-free diet since it has been demonstrated that when the fluid electrolytes are maintained within normal ranges in the adrenalectomized animal there are few deficiencies in carbohydrate metabolism (Long, Katzin and Fry 1940). Furthermore, several observers (Anderson *et al.*, 1940, Anderson 1943, Long *et al.*, 1940) have shown that the adrenalectomized rat chronically maintained on a high sodium chloride intake is capable of depositing liver glycogen at a normal rate. It is probable that in our experiments DCA was instrumental in maintaining electrolyte balance, thus allowing the animals to convert fed protein into carbohydrate but at a level somewhat lower than in those animals receiving whole extract. Animals on a carbohydrate-free diet and treated with DCA would be more fragile than those treated with cortical extract and, as was the case, fewer of them would be expected to survive the experimental period.

It is clear that the effectiveness of the preparations is to a certain extent dependent upon the method of administration because DCA

injected in suspension is more effective than when given in oil, and cortical extract is more beneficial when administered in drinking water than when injected. The superiority of a DCA suspension is also indicated by Sarason's work (1943), and others (e.g., Grollman, 1937) have found extracts more effective by mouth than by injection in chronic maintenance of adrenalectomized rats.

SUMMARY

A low carbohydrate diet greatly reduces the effectiveness of DCA, less so than it does whole extract of the adrenal cortex. This was demonstrated in treated adrenalectomized rats fed carbohydrate-deficient diets for periods of 10 and 20 days.

The effectiveness of the preparations was partially dependent upon the method of administration. Cortical extract (0.5 cc.) was more effective in the drinking water than when injected thrice daily, and much more effective than when injected once daily. DCA (0.5 mg.) was more effective when injected in an alcohol-water suspension than in oil, but regardless of the method of administration it was inferior to cortical extract given in drinking water.

The possible explanation of such results are discussed on the basis of the conversion of fed protein to carbohydrates under the influence of adrenal cortex hormones.

ACKNOWLEDGEMENT

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ADDENDUM

Since this paper was submitted for publication the author has corresponded with Dr. Albert Segaloff who has a paper in press showing that adrenalectomized rats with subcutaneous pellets of DCA grow and survive at a diminished rate when purified casein is used in a carbohydrate-free diet. Furthermore, he reports good growth and survival with DCA on a diet free from carbohydrate but containing crude casein.

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FURTHER STUDIES ON THE CHEMICAL NATURE OF COMPOUNDS WHICH INHIBIT THE FUNCTION OF THE THYROID GLAND[1]

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IN AN EARLIER STUDY 106 compounds were tested in rats for their capacity to interfere with the endocrine function of the thyroid gland. Each substance was administered to young rats by mixing it with the food or drinking water in various concentrations. At the end of ten days the thyroid glands were examined grossly and microscopically for evidence of hypertrophy and hyperplasia, and the relative activities of the compounds were then roughly compared on the basis of the minimal dose required to produce a noticeable effect (Astwood, 1943). Although this assay method was sufficiently reliable to detect wide differences in activity and to form the basis for the selection of compounds such as thiouracil for clinical trial, it suffered the handicap of subjective criteria and no quantitative dosage-response relationship could be established.

A more accurate and reproducible method based upon the weight and iodine concentration of the thyroid gland (Astwood & Bissell, 1944) has been used in the present investigation. The earlier experiments showed that of the compounds investigated the active ones could be considered to be derivatives either of thiourea or of aniline. A great many compounds had been tested before this relationship became clear and consequently relatively few thioureylene and aminobenzene derivatives were included. In the present investigation emphasis has been laid on compounds containing these or closely allied groupings in selecting substances for testing.

METHODS

The procedures employed differed somewhat from those previously reported (Astwood & Bissell, 1944). Young rats of either sex weighing 40 to 80 grams at the beginning of the experiment were used in groups of three animals each. The compounds to be tested were mixed with the food; this was found to be more satisfactory and to give more reproducible re-

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sults than administration in the drinking water. The rate of growth during the ten day feeding period was observed by weighing the animals every two days and was expressed as the average gain in grams per day. The total food consumed by the three animals during the ten day period was measured and from this the average daily intake of the compound per 100 grams of body weight was calculated using the average body weight of the group during the period of the test. The animals were killed with chloroform and the thyroid glands were weighed on a torsion balance; the weights of the glands were expressed in milligrams per 100 grams of body weight. The thyroid glands from each group of three animals were combined for a single iodine determination; the concentration of iodine was expressed in milli-

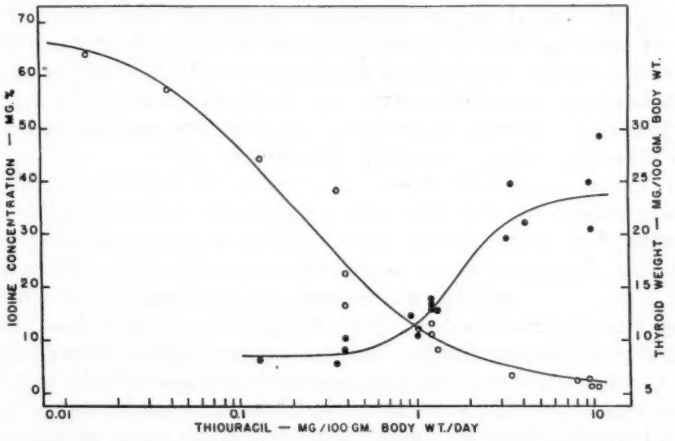


FIG. 1. Effect upon the weight (solid circles) and iodine concentration (open circles) of the thyroid gland of feeding various concentrations of thiouracil in ground Purina fox chow to young rats. The actual dose consumed daily, expressed in milligrams per 100 gm. body weight, is plotted on a log scale. Each point represents the value obtained with a single group of 3 rats.

grams per 100 grams of fresh tissue. With few exceptions the concentrations of compound in the diet were decimal fractions of 1% and 3%, providing doses which differed from one another by a factor of roughly three-fold over the entire dosage range. Some compounds were tested in only one or two doses while the more active ones and some which were of interest for reasons of chemical structure were tested over a wide range of dosage sometimes from 1% to 0.0001% of the diet.

Throughout these experiments thiouracil was used as a standard of reference and was considered to have an activity of 1.00; the relative anti-thyroid activities of other compounds could then be expressed numerically.

STANDARD DOSAGE-RESPONSE CURVES

Thiouracil was administered to 37 groups of rats in concentrations of from 0.0001 to 3% of the diet. The resulting weights and iodine concentrations of the thyroid glands were plotted against the loga-

rithm of the actual dose in mg. per 100 gm. of body weight per day. The resulting curves shown in Figure 1 were then used as a standard with which the responses from other compounds should be compared. The iodine concentration curve seemed best to fit a hyperbolic function and with increasing dosage the curve appeared to be asymptotic to a value of about 1 mg. of iodine per 100 gm. of thyroid tissue. Consequently the reciprocal of the iodine concentration minus one was plotted against the dose and a straight line was fitted to the points by eye. The method of least squares gave a poor fit because of the undue weight of the responses to the higher doses. The constants thus obtained were used to construct the curve reproduced in Figure 1 relating the actual iodine concentration to the dose. The dose was plotted on a log scale in order to condense the wide range within a reasonable space. The thyroid weight response could not be fitted to a simple curve and as the weight was found to be of use for quantitative purposes over only a restricted range it was not necessary to do more than fit it roughly to a sigmoid curve by eye.

It will be noted that thiouracil in a dose of 0.03 mg. per 100 gm. of body weight per day produced a significant decrease in the iodine concentration of the thyroid gland and that with increasing dosage the iodine concentration became proportionately lower. When a dose of 10.0 mg. was exceeded the iodine concentration fell to very low values which approached the limit of accuracy of the iodine method. For purposes of quantitative comparison the region between 40 and 5 mg.% of iodine corresponding roughly to dosages of 0.15 to 3.5 mg. of thiouracil proved to be most suitable. The useful range of the thyroid weight curve was more restricted and values outside of the region 10 to 18 mg. per 100 gm. of body weight corresponding to doses of 0.8 to 2.2 mg. of thiouracil were of no value beyond indicating that further tests were needed with larger or smaller doses.

Reference has been made to the fact that the shapes of the dosage-response curves of some compounds differed significantly from those of thiouracil (Astwood 1945b). The quantitative comparison of such compounds with thiouracil could only be made at some stipulated degree of response. For such purposes, and for the more exact comparison of some of the more interesting compounds, the response selected was an iodine concentration of 12 mg.% and a corresponding thyroid weight of 11.5 mg.%. This degree of response was induced by a dose of thiouracil averaging 1.07 mg. per 100 gm. of body weight per day.

There were several reasons for selecting this degree of response. It is a region which lies on the useful range of both curves, it is near the maximal end of one and the minimal region of the other and when comparison is made between a variety of compounds it has been observed that regardless of the disparity between the shapes of the dosage-response curves whenever the iodine concentration is de-

pressed to 12 mg.% there is a significant increase in thyroid weight. Furthermore there may be virtue in the fact that the dosage of thiouracil required to produce this degree of response would correspond, on a body weight basis to a daily dose of 0.64 gm. for a man of 60 kilograms, a figure which is not greatly in excess of the usual dose of thiouracil employed clinically.

In the tables to follow the compounds tested have been grouped as far as possible according to their chemical structure. The number of tests made with each substance is shown but in order to conserve space only representative experiments are recorded. The reliability of the values shown could not conveniently be expressed by a statistical calculation of the error, and the weight to be attached to them is indicated only by the number of tests performed.

It is to be emphasized that the estimates of activity are not precise and even when a large number of tests were performed the values shown for activity are not valid beyond one significant figure. In some instances a second figure is shown in order to indicate a difference in activity between compounds which were carefully compared with one another.

The compounds tested were derived from several sources and the origin of each is shown in the tables except for those substances which were purchased on the market. These were used without further identification or purification except in a few instances.

A rough measure of toxicity is provided by the degree of growth retardation in relation to the dose. It is to be pointed out that this index of toxicity is no more than a guide to wide differences in short term chronic toxicity, but it is useful in excluding a number of substances from more detailed study. Minor variations in the rate of weight gain were commonly observed in control animals and are of no toxicological significance. If, however, the rate of gain was reduced to 2 gms. per day or lower the compound was considered to be toxic in the dosage given. It appears that failure to eat (and consequently failure to gain) because of the taste of a compound can often be distinguished from failure to gain weight due to toxicity. In the former case abstention from food is usually confined to the first few days after which weight gain is resumed.

RESULTS

Thiourea derivatives. The 22 compounds tested which may be classified as simple derivatives of thiourea are listed in Table 1. Eight of these were found to possess significant antithyroid activity. Thiourea itself was one-eighth to one-tenth as active as thiouracil and its effectiveness was not modified significantly by the replacement of one, two or three of the hydrogen atoms by methyl groups. Of considerable interest from the structural point of view was the finding that when all four hydrogens were replaced by methyl groups the

TABLE 1. THIOUREAS

Compound	Formula (3. 2. 1. =N—C(=S)—N=)	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams**	Thyroid Weight mg./100 Gm. B.W.**	Thyroid Iodine Concentration mg./100 Gm.**	Estimated Activity
Thiourea	NH_2CSNH_2		4	7.0	2.3	9.0	22.9	
				49.	1.2	20.7	2.0	0.12
1-Methylthiourea	$NH_2CSNHCH_3$		2	9.5	1.5	15.1		0.1
1,3-Dimethylthiourea	$CH_3NHCSNHCH_3$	1	1	11.2	2.3	12.3	4.6	0.1
1,1,3-Trimethylthiourea	$CH_3NHCSN(CH_3)_2$	1	2	10.5	2.8	13.2	5.3	0.1
1,1,3,3-Tetramethylthiourea	$(CH_3)_2NCSN(CH_3)_2$	1	6	1.2	2.6	8.5	59.3	0.3
				13.9	1.2	16.8	1.9	
1,3-Diethylthiourea	$C_2H_5NHCSNHC_2H_5$		1	3.7	4.0	12.1	3.3	0.4
1-Isopropylthiourea	$NH_2CSNHCH(CH_3)_2$	2	3	1.6	2.9	9.7	33.6	0.4
				17.4	2.9	21.3	1.7	
1,3-Diisopropylthiourea	$(CH_3)_2CHNHCSNHCH(CH_3)_2$	1	2	1.2	2.5	7.7	36.2	0.3
1-Acetylthiourea	$NH_2CSNHCOCH_3$	3	1	8.6	−0.8	8.7	53.5	<0.01
1,3-Diacetylthiourea	$CH_3CONHCSNHCOCH_3$	3	1	7.7	−0.4	11.0	43.6	<0.01
4-Phenylthiosemicarbazide	$NH_2NHCSNHC_6H_5$	4	1	4.3	1.3	9.7	41.4	<0.03
1,3-Diaminothiourea (3-Thiocarbohydrazide)	$NH_2NHCSNHNH_2$	1	1	3.7	4.2	9.0		<0.03
1-(2-phenylureido)thiourea (4-Phenylcarbamyl-3-thiosemicarbazide)	$NH_2CSNHNHCONHC_6H_5$	1	2	38.0	4.4	8.9	35.6	<0.01
1-Sulfanilylthiourea	$NH_2CSNHSO_2C_6H_4NH_2$	1	2	130.0	3.0	9.4	38.2	<0.003
1,3-Bis(2-pyridyl)thiourea	$(C_5H_4N)NHCSNH(C_5H_4N)$	1	1	5.3	2.8	9.1	35.3	<0.03
1,3-Diheptylthiourea	$C_7H_{15}NHCSNHC_7H_{15}$	1	1	12.5	4.0	7.0	70.2	<0.01
1-Phenyl-3-dodecylthiourea	$C_{12}H_{25}NHCSNHC_6H_5$	1	1	12.6	3.1	7.5	70.0	<0.01
1,3-Bisdodecylthiourea	$C_{12}H_{25}NHCSNHC_{12}H_{25}$	1	1	10.9	3.0	8.1	74.6	<0.01
1,3-Diethyl-1,3-diphenylthiourea	$(C_6H_5)(C_2H_5)NCSN(C_2H_5)(C_6H_5)$	1	1	8.9	2.4	7.4	85.4	<0.01
2,5-Dithiobiurea (1,2-Bis(thiocarbamyl)hydrazine)	$NH_2CSNHNHCSNH_2$	1	2	27.9	2.6	9.0	45.1	<0.01
2-Ethylisothiourea sulfate	$[NH_2C(SC_2H_5)NH]_2 \cdot H_2SO_4$	1	2	54.0	2.5	10.1	40.6	<0.01
2-Benzhydrylisothiourea hydrobromide	$NH_2C[SCH(C_6H_5)_2]NH \cdot HBr$	4	1	23.0	1.6	9.2	63.9	<0.01

* 1. American Cyanamid Company 2. Dr. C. P. Richter 3. Dr. H. Christensen 4. Parke, Davis & Co.

** Control values of 12 groups of 3 animals each were: daily body weight gain 3.6 Gm. (2.7 to 5.1), thyroid weight in mg. per 100 Gm. body weight 8.06 (6.9 to 9.2), thyroid iodine concentration in mg. per 100 Gm. of wet tissue 73.1 (58.8 to 80.3).

activity was considerably enhanced. The high activity of tetramethylthiourea is shared by the diethyl, *iso*propyl, and di*iso*propyl derivatives and thus far no open chain thiourea has been found which exceeds these compounds in potency.

When the substituents on the thiourea molecule are large the activity is markedly decreased or lost. A marked decrease or loss of potency also seems to be associated with the presence of polar groups such as amino, imino, or carbonyl in the substituents attached to one or both nitrogen atoms. Thioureas which are of necessity in the pseudo or iso configuration by virtue of substitution on the sulfur atom have no appreciable antithyroid activity. These generalizations are quite analogous to those which may be drawn from the extensive series of tests on thiouracil derivatives cited below.

A striking finding was the complete inactivity of dithiobiurea, a compound containing two complete thiourea molecules, and of sulfanilylthiourea a compound in which two different types of active grouping are united; as most of the N substituted sulfonamides are active the thiourea derivative would have been expected to have been active by virtue either of the aminobenzene grouping or the thiourea group.

The toxicities of the more active thioureas did not differ widely but tended to parallel the biological activity and to be more toxic than thiouracil. Among the inactive compounds the most toxic were the two acetyl derivatives.

Imidazoles and imidazolines. The inclusion of a thioureylene group in a five membered heterocyclic ring structure as in 2-mercaptoimidazole results in compounds which are in some instances more active than thiourea itself. Of the 10 compounds of this type listed in Table 2, 2-mercaptoimidazoe was the most active substance encountered—one and one half times as active as thiouracil. The dihydro derivative, mercaptoimidazoline (ethylenethiourea) and 2-thiohydantoin were less than one half as active as thiouracil and slightly less active than 2-mercaptobenzimidazole (phenylene thiourea). Again, as in the case of the straight chain thioureas large substituents, additional polar groups, or substitution on the sulfur atom all tended to decrease antithyroid potency. With the possible exception of 2-mercaptoimidazole, the toxicities of these compounds were higher than the thioureas of comparable potency. Larger doses of 2-mercaptoimidazoline, 2-mercaptobenzimidazole and the 2-thiohydantoins than those shown in Table 2 completely prevented growth.

Thiazoles, thiazolines and oxazolines. The basic difference between this series of compounds and the foregoing is the replacement of one of the nitrogen atoms of the thioureylene grouping by a sulfur or an oxygen atom. It is apparent that this change is not incompatible with antithyroid potency suggesting that the structural unit essential to

TABLE 2. IMIDAZOLES AND IMIDAZOLINES

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
2-Mercaptoimidazole	NHC(SH)NCHCH	1	4	0.13	4.9	7.8	47.4	
				0.93	2.5	12.3	3.1	1.5
				11.2	2.6	22.1	2.8	
4,5-Diphenyl-2-mercaptoimidazole	NHC(SH)NC(C_6H_5)C(C_6H_5)	2	2	17.6	1.3	10.2	41.7	0.03
4-β-hydroxyethyl-2-mercaptoimidazole	NHC(SH)NC(CH_2CH_2OH)CH	1	1	7.5	1.1	8.4	89.9	0.01
2-Mercaptoimidazoline	NHC(SH)NCH_2CH_2	1	2	1.2	4.0	8.0	18.7	0.4
2-Thiohydantoin	NHC(S)NHC(O)CH_2	1	2	1.4	4.9	8.3	13.7	0.4
5,5-Diphenyl-2-thiohydantoin	NHC(S)NHC(O)C(C_6H_5)$_2$	2	1	5.9	2.4	14.7	26.3	0.5
5,5-Diphenyldithiohydantoin	NHC(S)NHC(S)C(C_6H_5)$_2$	2	1	6.0	1.4	9.2	49.7	<0.1
2-Thioparabanic acid	NHC(S)NHC(O)C(O)	1	1	3.6	2.9	6.7	71.8	<0.03
2-Mercaptobenzimidazole	NHCSHNC_6H_4	2	5	1.5	3.8	12.6	22.0	0.5
				10.7	0.8	27.1	2.2	
2-Benzylthioimidazoline	NHC($SCH_2C_6H_5$)NCH_2CH_2	2	2	17.6	1.5	10.3	30.0	<0.001

* 1. American Cyanamid Company 2. Parke, Davis & Co.

activity is a=N—C(=S)—X group where X is an N, O, or S atom and not necessarily an entire thioureylene radical. Three compounds were tested which possess no free sulfur atom; 2-aminothiazole and its 4-methyl derivative were active even though the sulfur was a part of the ring structure while 2-aminothiazoline was inert. The introduction of two hydrogen atoms to saturate the 4,5 double bond thus decreased the biological activity. The opposite of this phenomenon was observed in the case of the 2-mercapto derivatives; 2-mercaptothiazoline was found to be more active than thiouracil while all of the simple derivatives of 2-mercaptothiazole were of very low activity.

An inspection of the data of Table 3 shows that the factors which modify activity in these types of compound differ greatly from those noted above with the thioureylene derivatives. The activity of 2-mercaptothiazoline was decreased by all of the alkyl substitutions which were tried; the activity of 2-mercaptobenzothiazole was just detectable in contrast to the high potency of the analogous compound of the imidazole series, 2-mercaptobenzimidazole; polar groups did not necessarily decrease potency in the members of this series, for 2-mercaptobenzothiazole was less active than its 6-amino derivative.

The active compounds shown in Table 3 were on the whole quite toxic. The increased toxicity of 2-mercaptothiazoline over that of thiouracil was quite out of proportion to its increased potency.

Miscellaneous heterocyclic compounds. Listed in Table 4 are 17 mercapto or thio derivatives of 5 or 6 membered heterocyclic ring compounds. 3-Mercapto-1,2,4-triazole was one-third as active as thiouracil; each of the 5 derivatives tested possessed additional polar groups and none was active in the dosages tested. Only two thiadiazoles were assayed; one of these, 5-amino-2-mercapto-1,3,4-thiadiazole, was distinctly more active than thiouracil and as far as can be determined from the data, no more toxic than thiouracil.

The distinct activity of 5,6-dihydro-2-mercapto-1,3,4-thiazine is interesting in that replacement of the sulfur by nitrogen, as in tetrahydro-2-thiopyrimidone shown below, does not appreciably change the activity. This type of analogy fails to hold, however, with propiorhodanine. This substance was not active though it might be considered a sulfur analogue of dihydro-2-thiouracil, a distinctly active compound. It will be recalled that the substitution of one nitrogen atom in 2-mercaptoimidazoline by a sulfur atom to form 2-mercaptothiazoline was attended by a considerable increase in potency. It was therefore contrary to expectation and disappointing to find that propiorhodanine was so much less active than the corresponding pyrimidine.

The two thiadiazines and two triazines tested were either of very

TABLE 3. THIAZOLES, THIAZOLINES AND OXAZOLINES

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
2-Aminothiazole	$SC(NH_2)NCHCH$	1	2	10.8	−1.4	12.9	6.2	0.15
2-Amino-4-methylthiazole	$SC(NH_2)NC(CH_3)CH$		1	11.1	1.0	12.8	7.9	0.15
2-Aminothiazoline	$SC(NH_2)NCH_2CH_2$	1	1	22.5	−0.7	8.6	84.2	<0.003
2-Amino-4-oxo-5-thiazolineacetic acid (Pseudothiohydantoin acetic acid)	$SC(NH_2)NC(O)CHCH_2COOH$	2	1	7.3	2.7	7.7	67.2	<0.01
4-Methyl-2-mercaptothiazole	$SC(SH)NC(CH_3)CH$	1	1	3.9	3.8	7.4	42.4	<0.1
4,5-Dimethyl-2-mercaptothiazole	$SC(SH)NC(CH_3)C(CH_3)$	3	2	36.3	2.5	9.4	37.7	<0.01
4-Phenyl-2-mercaptothiazole	$SC(SH)NC(C_6H_5)CH$	3	2	30.9	2.7	8.5	60.8	<0.01
2-Mercaptobenzothiazole	$SC(SH)NC_6H_4$	1	2	161.	3.6	13.7	17.6	0.005
6-Amino-2-mercaptobenzothiazole	$SC(SH)NC_6H_3NH_2$		1	20.1	−0.1	15.0	6.0	0.08
2-Mercaptothiazoline	$SC(SH)NCH_2CH_2$	1	5	.34 1.5 8.0	2.6 3.1 1.4	8.0 14.8 25.7	28.5 8.8 1.1	1.3
4,4-Dimethyl-2-mercaptothiazoline	$SC(SH)NC(CH_3)_2CH_2$	1	1	7.5	0.7	10.7	23.4	0.05
5,5-Dimethyl-2-mercaptothiazoline	$SC(SH)NCH_2C(CH_3)_2$	1	2	1.0	2.6	6.3	17.5	0.3
4-Ethyl-2-mercaptothiazoline	$SC(SH)NCH(C_2H_5)CH_2$	1	2	10.0	1.6	10.2	24.9	0.03
3-Phenyl-2-thiothiazolidone	$SC(S)N(C_6H_5)CH_2CH_2$	4	2	19.0	0.9	8.1	78.4	<0.003
4-p-Dimethylaminobenzalrhodanine	$SC(S)NHC(O)CCHC_6H_4$-p-$N(CH_3)_2$		1	5.7	1.9	8.2	33.5	0.04
2-Mercaptoöxazoline	$OC(SH)NCH_2CH_2$	1	1	10.0	1.9	17.2	5.6	0.2
5,5-Dimethyl-2-mercaptoöxazoline	$OC(SH)NCH_2C(CH_3)_2$	1	2	1.1	3.5	11.2	21.6	0.4
4-Ethyl-5-isobutyl-2-mercaptoöxazoline	$OC(SH)NCH(C_2H_5)CHCH_2CH(CH_3)_2$	3	2	17.7	−0.3	10.5	48.5	<0.01

* 1. American Cyanamid Company 2. E. R. Squibb & Sons 3. Parke, Davis & Co. 4. Abbott Laboratories

TABLE 4. OTHER HETEROCYCLIC COMPOUNDS

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
3-Mercapto-1,2,4-triazole	NNHC(SH)NCH	1	2	8.8	2.1	16.5	5.3	0.3
				3.8	5.0	9.2	9.8	
5-Hydroxy-3-mercapto-1,2,4-triazole (Thiourazole)	NNHC(SH)NCOH	1	1	11.5	3.3	8.7	50.5	<0.01
4-Formyl-3-mercapto-1,2,4-triazole	NNC(SH)N(CHO)CH	1	1	5.9	−2.7	6.9	104.	<0.01
4-Phenyl-5-hydroxy-3-mercapto-1,2,4-triazole	NNC(SH)N(C_6H_5)COH	1	1	12.0	3.6	8.5	67.7	<0.01
5-Amino-3-mercapto-1,2,4-triazole	NNHC(SH)NCNH_2	1	1	11.6	2.7	9.0	48.4	<0.01
3,5-Dimercapto-1,2,4-triazole	NNHC(SH)NCSH	1	1	11.1	3.4	7.8	47.5	<0.01
5-Amino-2-mercapto-1,3,4-thiadiazole	SC(SH)NNCNH_2	1, 2		0.4	5.6	7.4	24.8	
			3	1.1	3.5	15.9	7.6	1.2
				9.7	2.2	26.5	1.4	
2,5-Dimercapto-1,3,4-thiadiazole	SC(SH)NNCSH	1	2	30.3	3.0	10.5	30.3	<0.03
2-Thio-1,3-dithiolane	SC(S)SCH_2CH_2	3	2	4.8	−4.7	8.5	97.0	<0.003
7-Thio-1,4-diazabicyclo(2,2,1)heptane	NCH_2CH_2NCH_2CH_2 C(S)	3	2	23.1	1.7	9.0	58.7	<0.01
5,6-Dihydro-2-mercapto-1,3,4-thiazine	SC(SH)N$CH_2CH_2CH_2$	1	2	12.1	2.1	16.6	2.3	0.51
4-Oxo-5,6-dihydro-2-mercapto-1,3,4-thiazine (Propiorhodanine)	SC(SH)NCOCH_2CH_2	1	2	67.0	2.9	8.9	39.0	<0.01
4,6-Diamino-2-thio-1,3,5-thiadiazine	SC(S)NC(NH_2)NCNH_2	1	2	203.	1.7	13.1	19.0	0.005
4,6-Dimethyltetrahydro-2-mercapto-1,3,5-thiadiazine (Carbothialdine)	SC(SH)NCH(CH_3)NHCHCH_3		1	14.0	3.1	10.4	45.6	<0.01
4,6-Diamino-2-mercapto-*s*-triazine (Thioammeline)	NC(SH)NC(NH_2)NC(NH_2)	1, 2	1	22.9	3.6	8.1	90.9	<0.01
4,6-Diamino-2-carbamylmethylthio-*s*-triazine	NC(SCH_2CONH_2)NC(NH_2)NCNH_2	2	1	22.4	3.9	8.1	74.4	<0.01
2-Mercaptopyrazine	NC(SH)CHNCHCH	1	1	12.5	1.7	8.7	65.6	<0.01

* 1. American Cyanamid Company 2. Parke, Davis & Co. 3. Abbott Laboratories

TABLE 5. MISCELLANEOUS SULFUR COMPOUNDS

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
Thioglycollic acid	$HSCH_2COOH$		1	9.9	−3.2	10.0	155.0	<0.01
Cysteine	$HSCH_2CH(NH_2)COOH$	1	2	45.0	4.0	8.6	78.8	<0.003
2-Mercaptoethylamine	$HSCH_2CH_2NH_2$	2	1	11.7	3.2	8.6	66.1	<0.01
Methylisothiocyanate	$SCNCH_3$		2	73.	0.4	9.7	43.6	<0.01
Thioacetamide	NH_2CSCH_3		2	7.6	−1.9	7.5	84.2	<0.01
Thiopropionamide	$NH_2CSCH_2CH_3$		1	4.5	0.9	6.3	55.8	<0.03
Benzylthiothioacetamide	$NH_2CSCH_2SCH_2C_6H_5$	1	1	8.2	5.5	7.7	53.4	<0.03
Trimethyldithiocarbamate	$(CH_3)_2NC(S)SCH_3$	1	1	7.3	−0.1	9.4	89.2	<0.01
Dimethylammoniumdimethyldithiocarbamate	$(CH_3)_2NC(S)SNH_2(CH_3)_2$		2	9.5	2.0	10.4	41.9	0.01
Thiocarbamylthioglycollic acid	$NH_2C(S)SCH_2COOH$		6	1.2 5.9	4.2 1.2	10.5 27.0	17.3 2.1	0.4
Tetramethylthiuramsulfide	$(CH_3)_2NC(S)S(S)CN(CH_3)_2$	1	1	9.1	1.0	9.4	80.3	<0.01
Tetramethylthiuramdisulfide	$(CH_3)_2NC(S)SS(S)CN(CH_3)_2$	1	1	6.1	0	10.6	70.2	<0.01
9-(10)-Acridinethione	$C_6H_4C(S)C_6H_4NH$	1	1	30.0	2.2	8.1	46.6	<0.003
Tetra-(4-pyridyl)-thiophene	$SC(C_5H_4N)C(C_5H_4N)C(C_5H_4N)C-C_5H_4N$		1	27.6	1.6	10.1	51.8	<0.003
Ethyl-2-benzothiazylsulfone	$C_2H_5SO_2CNC_6H_4S$	3	1	13.0	3.7	8.2		0.01

1. American Cyanamid Company 2. Abbott Laboratories 3. Parke, Davis & Company

low potency or inert. 2-Mercaptopyrazine had no activity in the dosage tested.

Other sulfur compounds. The fifteen compounds listed in Table 5 are related in one way or another to those shown in Tables 1 to 4. It may be seen that several compounds possessing a sulfhydryl group were inactive and that even such substances as thioacetamide, thiopropionamide, methylisothiocyanate and trimethyldithiocarbamate were inert. The dithiocarbamates and the thiuram derivatives are closely related structurally to the 2-mercaptothiazoles but their antithyroid activity was found to be very low or undetectable. It is of considerable interest though that thiocarbamylthioglycollic acid was nearly one half as active as thiouracil. This compound is the only highly active straight chain thiocarbonamide thus far encountered which does not possess the entire thioureylene grouping. It may be considered an open chain analogue of the 2-mercaptothiazoles and it is known to lose one molecule of water on heating and to form the ring structure rhodanine.

Thiopyrimidones. The simplest 6 membered cyclic derivatives of thiourea are the 2-thiopyrimidones; eight of these and one 4-thio derivative are listed in Table 6. None was found to be significantly active except the tetrahydro derivative which, as mentioned above, resembles the corresponding 1,3,4-thiazine in biological activity. Substituent methyl groups on 2-thiopyrimidone decreased activity and polar groups abolished it altogether. In view of the high activity to be found among derivatives of thiouracil and thiobarbituric acid a further exploration of the thiopyrimidones would be of interest.

Thiouracils. The largest series of compounds tested were the thiouracils. These derivatives provided the best opportunity of any to relate structure to activity and within this group are the most highly active antithyroid compounds yet encountered. Most of them were synthesized especially for this study by Anderson, Halverstadt, Miller and Roblin (1945). Relatively minor structural alterations have been found either to increase potency as much as ten fold or to reduce it to undetectable proportions. At the extremes, a difference in potency of at least 10,000 fold is determined by a change from an amino to a hydrocarbon group on carbon atom 6 of the thiouracil molecule.

Several generalizations emerge from a study of the data in Table 7. Dihydrothiouracil is less than one tenth as active as thiouracil, indicating the importance of the double bond. Substitution on the nitrogen atoms of methyl or ethyl groups appears to decrease potency, more so on nitrogen 3 than on the nitrogen at position 1. Unfortunately no compounds were available for testing in which the hydrogen atoms at positions 1 and 3 were both replaced.

Alkyl groups on the carbon atom at position 5 had a distinct effect on activity. 5-Methylthiouracil was less active but 5-ethyl was about

TABLE 6. THIOPYRIMIDONES

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
Tetrahydro-2-thiopyrimidone	$NHC(S)NHCH_2CH_2CH_2$	1	2	11.8	3.1	13.1	6.9	0.13
1-Butyltetrahydro-2-thiopyrimidone	$C_4H_9NC(S)NHCH_2CH_2CH_2$	2	2	19.4	−1.5	11.4	31.2	0.01
5-Chloro-2-thiopyrimidone	$NHCSNCHC(Cl)CH$	1	1	8.4	1.3	8.5	69.6	<0.01
4,6-Dimethyl-2-thiopyrimidone	$NHCSNC(CH_3)CHCCH_3$	1	2	237.	1.4	14.9	9.2	0.01
1,4,6-Trimethyl-2-thiopyrimidone	$CH_3NC(S)NC(CH_3)CHCCH_3$	1	2	254.	0.8	17.9	5.5	0.02
4-Amino-5-carboxy-2-thiopyrimidone	$NHC(S)NC(NH_2)C(COOH)CH$	1	2	145.	3.9	9.9	36.3	<0.003
4-Amino-5-carbethoxy-2-thiopyrimidone	$NHC(S)NC(NH_2)C(COOC_2H_5)CH$	1	2	131.	3.4	10.4	36.8	<0.003
4,6-Diamino-2-thiopyrimidone	$NHC(S)NC(NH_2)CHCNH_2$	3	2	83.3	2.2	7.9	100.0	<0.003
2-Amino-4-thiopyrimidone	$NHC(NH_2)NC(S)CHCH$	1	1	11.4	2.1	7.0	43.6	<0.01

* 1. American Cyanamid Company 2. Abbott Laboratories 3. Parke, Davis & Co.

3.5 times as active as thiouracil. As the length of the alkyl chain increased, potency dropped off rapidly and even the 5-*iso*propyl derivative was less active than 5-ethylthiouracil. The *n*-butyl derivative was less active than the parent compound and further exploration of this series was not undertaken.

Substitution of the hydrogen atom at position 6 by hydrocarbon radicles was attended by more striking effects. At this position a methyl group did not alter activity, an ethyl or *iso*propyl increased it many fold and the maximal activity was reached with the *n*-propyl derivative which was found to be 11 times as active as thiouracil. With longer hydrocarbon chains the activity declined progressively in the order *tert.* butyl, *sec.* butyl, *iso*butyl, *n*-butyl and amyl; the last was similar in potency to the cyclohexyl derivative and just slightly more active than thiouracil itself. 6-*n*-Hexylthiouracil was only about one fifth as active as the parent substance.

The aryl substituents on carbon atom 6 exhibited a very restricted range of potentiation. The phenyl and phenethyl derivatives differed little from thiouracil while the intermediate compound 6-benzylthiouracil was ten times as active. The very low potency of 6-(p-chlorophenyl)-2-thiouracil is an unusual finding.

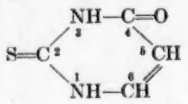
The five compounds substituted at both positions 5 and 6 indicated that further potentiation could not be induced by this means. It appeared that a methyl or an ethyl group at position 5 actually decreased the degree of enhancement provided by an appropriate substituent at position 6.

In contrast to the potentiation provided by hydrocarbon substituents at positions 5 or 6 are the observations on the influence of other groups in these positions. A complete loss of activity followed the replacement of the hydrogen atom at positions 5 or 6 by amino, carboxy, carbethoxy, or cyano groups suggesting that loss of activity is associated with the polar nature of the substituents. As noted below an additional hydroxyl group at position 6 of thiouracil (2-thiobarbituric acid) abolished activity but appropriate hydrocarbon substituents on position 5 then restored it.

As in the other instances already mentioned substitution on the sulfur atom of 2-thiouracil abolished the antithyroid effect.

The toxicity of the highly active thiouracil derivatives is of considerable importance and should be the subject of more detailed study. The data shown in Table 6 indicates that on a weight basis these more potent agents cause a greater retardation of growth than equal amounts of thiouracil. It may be seen that one third to one-fourth as much 6-ethyl, 6-*iso*propyl or 6-*n*-propyl thiouracil was required to induce a retardation of growth as was required with thiouracil. In view of the much higher activity of these derivatives they are therefore much less toxic than thiouracil when given in doses of equivalent antithyroid effectiveness. The inactive thiouracil derivatives were on the whole less toxic than thiouracil itself.

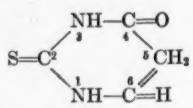
TABLE 7. THIOURACILS

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
2-Thiouracil		1	37	0.04	4.1	7.6	56.3	
				0.13	4.3	7.6	44.0	
				0.4	4.0	8.6	25.6	
				1.2	3.1	11.9	11.4	1.00
				3.6	3.0	21.4	4.4	
				10.0	2.3	23.4	1.9	
				101.0	1.7	25.4	1.4	
Dihydrothiouracil	(5)-H_2 (6)-H_2	2	3	7.7	2.3	10.8	19.8	0.08
				43.0	1.2	13.7	4.1	
1-Methylthiouracil	(1)-CH_3	1	2	4.0	2.5	17.9	6.2	0.5
1-Ethylthiouracil	(1)-CH_2CH_3	1	1	1.2	3.5	10.7	18.8	0.7
1-Benzylthiouracil	(1)-$CH_2C_6H_5$	1	4	1.4	3.5	12.1	18.6	0.7
				116.0	0.7	33.7	3.6	
3-Methylthiouracil	(3)-CH_3	1	2	6.1	2.7	14.0	9.5	0.3
3-Ethylthiouracil	(3)-CH_2CH_3	1	2	14.4	3.1	12.2	18.1	0.2
5-Methylthiouracil	(5)-CH_3	3	2	1.5	4.0	10.7	12.1	0.7
5-Ethylthiouracil	(5)-CH_2CH_3	1	4	0.4	4.1	12.6	9.5	3.5
				24.	0.5	28.2	2.7	
5-*n*-Propylthiouracil	(5)-$CH_2CH_2CH_3$	1	2	0.4	5.3	10.9	11.6	2.0
5-*Iso*propylthiouracil	(5)-$CH(CH_3)_2$	1	2	1.2	4.6	22.8	3.9	2.5
5-*n*-Butylthiouracil	(5)-$CH_2CH_2CH_2CH_3$	1	1	1.3	3.6	10.9	19.8	0.6
6-Methylthiouracil	(6)-CH_3	1, 3	3	1.3	3.7	11.9	8.8	1.
6-Ethylthiouracil	(6)-CH_2CH_3	1	18	0.04	3.2	10.2	30.2	
				0.13	3.9	9.9	16.0	
				0.4	3.1	19.6	4.0	
				1.2	2.7	28.3	1.6	8
				4.0	2.5	36.2	0.9	
				27.8	2.0	28.8	1.4	
6-*n*-Propylthiouracil	(6)-$CH_2CH_2CH_3$	1	15	0.06	5.3	8.1	20.3	
				0.13	4.8	11.8	5.6	
				3.2	2.9	28.6	1.4	11
				10.9	2.9	24.3	1.3	
				26.1	0.8	21.8	2.6	

6-*Iso*propylthiouracil	(6)-$CH(CH_3)_2$	1	4	0.12	4.7	10.6	9.7	
				1.1	3.0	29.3	1.6	9
				29.0	1.5	17.2	5.0	
6-*n*-Butylthiouracil	(6)-$CH_2CH_2CH_2CH_3$	1	4	0.12	3.6	7.7	36.6	3
				1.3	3.9	23.1	2.5	
6-*Iso*butylthiouracil	(6)-$CH_2CH_2(CH_3)_2$	1	6	0.14	4.2	9.3	17.0	5
				1.1	3.0	25.2	1.4	
6-*Sec.* Butylthiouracil	(6)-$CH(CH_3)CH_2CH_3$	1	6	0.14	3.9	8.8	15.9	6
				1.1	3.1	16.1	2.9	
6-*Tert.* Butylthiouracil	(6)-$C(CH_3)_3$	1	5	0.14	4.0	14.7	8.4	9
				1.1	3.1	19.2	5.2	
6-*n*-Amylthiouracil	(6)-$CH_2CH_2CH_2CH_2CH_3$	1	2	1.3	3.9	13.0	7.3	1.3
6-*n*-Hexylthiouracil	(6)-$CH_2CH_2CH_2CH_2CH_2CH_3$	1	2	3.9	3.9	9.4	17.4	0.18
6-Cyclohexylthiouracil	(6)-$CHCH_2CH_2CH_2CH_2CH_2$	1	2	1.4	3.6	14.4	9.7	1.2
6-Phenylthiouracil	(6)-C_6H_5	1	2	1.2	4.2	12.1	9.4	1.
6-(*p*-Chlorophenyl)thiouracil	(6)-C_6H_4Cl	1	3	13.2	3.6	6.9	46.4	<0.01
6-Benzylthiouracil	(6)-$CH_2C_6H_5$	1	6	0.14	3.3	12.2	8.5	10
				1.2	3.6	22.1	2.3	
6-Phenethylthiouracil	(6)-$CH_2CH_2C_6H_5$	1	2	1.4	3.0	13.0	8.5	1.2
6-Trifluoromethylthiouracil	(6)-CF_3	1	2	13.9	4.0	10.0	69.8	<0.01
5,6-Dimethylthiouracil	(5)-CH_3, (6)-CH_3	1	2	1.3	3.3	16.6	10.0	1.2
5-Ethyl-6-methylthiouracil	(5)-CH_2CH_3, (6)-CH_3	1	2	1.3	3.0	14.9	12.1	0.9
5-Methyl-6-ethylthiouracil	(5)-CH_3, (6)-CH_2CH_3	1	4	0.4	3.8	12.3	9.4	
				1.4	3.4	20.8	5.7	3.5
				10.0	2.9	23.8	2.1	
5,6-Diethylthiouracil	(5)-CH_2CH_3, (6)-CH_2CH_3	1	4	0.4	3.8	10.5	20.2	
				1.4	2.9	24.9	5.2	2
				9.3	1.8	25.9	3.2	
5,6-Trimethylenethiouracil	(5,6)-$CH_2CH_2CH_2$-	1	2	4.1	3.9	11.5	10.7	0.3
5-β-Hydroxyethyl-6-methylthiouracil	(5)-CH_2CH_2OH, (6)-CH_3	1	1	3.9	3.6	8.8	64.9	<0.01
2-Thioxanthine	(5,6)-NHCHN-	1	2	68.	3.6	8.3	58.4	<0.003
6-Aminothiouracil	(6)-NH_2	1, 4	6	83.	3.3	8.9	35.0	<0.001
6-Carboxythiouracil (Thioorotic acid)	(6)-COOH	1	2	91.	3.8	7.5	64.0	<0.001
6-Amino-5-nitrosothiouracil	(5)-NO, (6)-NH_2	4	1	25.4	4.4	7.5	81.5	<0.003
5-Cyanothiouracil	(5)-CN	1	2	127.	4.2	8.8	63.0	<0.001
5-Carbethoxythiouracil	(5)-$COOC_2H_5$	1	2	140.	2.3	8.3	51.8	<0.001
4-Thiouracil	(2) = O, (4) = S	1	1	50.	2.5	13.1	10.1	0.03
Dithiouracil	(2) = S, (4) = S	1	4	0.34	3.0	9.3	39.0	0.9
				14.8	1.7	20.5	1.7	
5-Methyl-2-ethylthiouracil	(2)-SC_2H_5, (5)-CH_3	3, 4	1	13.4	4.0	8.6	38.7	<0.03
6-Methyl-2-ethylthiouracil	(2)-SC_2H_5, (6)-CH_3	4	1	23.4	3.8	7.5	32.4	<0.03
5-Carbethoxy-2-ethylthiouracil	(2)-SC_2H_5, (5)-$COOC_2H_5$	3	1	12.8	4.4	8.1	88.6	<0.03
4-Thio-2-methylthiouracil	(2)-SCH_3, (4) = S	1	1	6.6	2.2	9.7	56.2	<0.03

* 1. American Cyanamid Company 2. E. R. Squibb 3. Dr. T. B. Johnson 4. Parke, Davis & Co

TABLE 8. THIOBARBITURIC ACIDS

Compound	Formula		Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
									
C-5 Substituted Thiobarbituric acids	Substituents on C-5								
2-Thiobarbituric acid	-H	-H		3	208.	2.1	7.7	49.2	<0.003
5-Ethyl-	-H	$-C_2H_5$	1	2	123.	3.3	7.9	63.4	<0.003
5,5-Dimethyl-	$-CH_3$	$-CH_3$	1	1	8.2	3.5	12.8	18.0	0.1
5-Methyl-5-ethyl-	$-CH_3$	$-C_2H_5$	1	1	8.5	3.1	12.3	7.2	0.1
5,5-Diethyl-	$-C_2H_5$	$-C_2H_5$	1, 2	9	0.4	3.4	10.2	24.2	
					5.9	2.7	16.3	3.6	1.7
					57.9	2.2	22.7	1.8	
5-Ethyl-5-isopropyl-	$-C_2H_5$	$-CH(CH_3)_2$	1	2	1.3	3.2	13.9	12.8	1.
5,5-Diallyl-	$-CH_2CHCH_2$	$-CH_2CHCH_2$	1	5	1.2	2.7	14.3	11.8	1.
					10.6	2.7	13.9	6.7	
5-Ethyl-5-β-hydroxyethyl-	$-C_2H_5$	$-CH_2CH_2OH$	1	1	12.4	3.2	10.1	34.5	0.01
5-Isoamyl-5-β-hydroxyethyl-	$-C_5H_{11}$	$-CH_2CH_2OH$	1	1	14.2	3.0	9.2	58.4	<0.01
5-Ethyl-5-α-ethylthioethyl-	$-C_2H_5$	$-CH(CH_3)SC_2H_5$	3	2	3.2	3.7	12.5	30.2	0.2
5-Ethyl-5-isopropylthiomethyl-	$-C_2H_5$	$-CH_2SC_3H_7$	3	2	4.0	3.9	15.8	16.0	0.1
5-n-Propyl-5-α-ethylthioethyl-	$-C_3H_7$	$-CH(CH_3)SC_2H_5$	3	2	3.4	3.6	12.0	39.8	0.1
5-Ethyl-5-α-isoamylthioethyl-	$-C_2H_5$	$-CH(CH_3)SC_5H_{11}$	3	1	32.7	2.3	17.3	6.3	0.07
5-Ethyl-5-α-n-butylthioethyl-	$-C_2H_5$	$-CH(CH_3)SC_4H_9$	3	2	31.8	2.1	19.3	7.4	0.07
5-Allyl-5-β-ethylthioethyl-	$-CH_2CHCH_2$	$-CH_2CH_2SC_2H_5$	3	2	25.2	2.8	18.7	13.2	0.07
5-Ethyl-5-isoamylthiomethyl-	$-C_2H_5$	$-CH_2SC_5H_{11}$	3	1	33.6	3.1	15.9	9.9	0.05
5-Ethyl-5-α-n-amylthioethyl-	$-C_2H_5$	$-CH(CH_3)SC_5H_{11}$	3	1	35.1	3.3	17.5	11.0	0.05
5-Ethyl-5-α-ethylthio-n-butyl-	$-C_2H_5$	$-CH(C_3H_7)SC_2H_5$	3	1	31.8	1.8	17.8	10.1	0.05
5-Isobutyl-5-ethylthiomethyl-	$-C_4H_9$	$-CH_2SC_2H_5$	3	1	26.7	1.8	12.9	14.5	0.04
5-n-Butyl-5-n-butylthiomethyl-	$-C_4H_9$	$-CH_2SC_4H_9$	3	1	35.7	3.2	13.8	14.6	0.04
5-Allyl-5-β-n-propylthioethyl-	$-CH_2CHCH_2$	$-CH_2CH_2SC_3H_7$	3	1	36.0	3.5	13.9	15.1	0.04
5-Allyl-5-β-isopropylthioethyl-	$-CH_2CHCH_2$	$-CH_2CH_2SC_3H_7$	3	1	36.3	2.5	15.0	13.9	0.04
5-Ethyl-5-α-isopropylthiopropyl-	$-C_2H_5$	$-CH(C_2H_5)SC_3H_7$	3	1	24.3	0.1	14.9	13.7	0.04
5-Isobutyl-α-allylthioethyl-	$-C_4H_9$	$-CH(CH_3)SC_3H_5$	3	1	30.9	2.5	17.3	32.4	0.03
5-Allyl-5-β-n-butylthioethyl-	$-CH_2CHCH_2$	$-CH_2CH_2SC_4H_9$	3	2	34.2	2.6	12.1	30.2	0.02

5-*n*-Butyl-5-*β*-allylthioethyl-	$-C_4H_9$	$-CH_2CH_2SC_3H_5$	3	1	39.9	4.0	9.9	44.6	0.01
5-Isoamyl-5-*β*-ethylthioethyl-	$-C_5H_{11}$	$-CH_2CH_2SC_2H_5$	3	1	32.1	3.1	9.7	38.2	0.01
Other Barbituric acids	Substituents								
5-Ethyl-5-isoamyl-2,4-dithiobarbituric acid	(2,4) = S, (5)-C_2H_5, (5)-C_5H_{11}		2	1	1.4	3.4	7.1	65.6	<0.03
5,5-Diethyl-2,4,6-trithiobarbituric acid	(2,4,6) = S, (5)-C_2H_5, (5)-C_2H_5		4	2	11.6	0.6	18.5	13.2	0.1
5-(1-methylbutyl)-4-imino-2-thiobarbituric acid	(2) = S, (4) = NH, (5)-$CH(CH_3)$-C_3H_7		1	2	88.5	0.8	9.0	81.0	<0.003
5-Methyl-5-(1-methylbutyl)4-imino-2 thiobarbituric acid	(2) = S, (4) = NH, (5)-CH_3, (5)-$CH(CH_3)C_2H_7$		1	1	11.6	3.0	11.5	18.7	0.07
5-Ethyl-5-butylthiomethylbarbituric acid	(2) = O, (5)-C_2H_5, (5)-$CH_2SC_4H_9$		3	1	31.8	2.8	8.7	63.7	<0.003
5-Ethyl-5-sec. butylthiomethylbarbituric acid	(2) = O, (5)-C_2H_5, (5)-$CH_2SC_4H_9$		3	1	28.8	2.3	9.1	70.5	<0.003
5-Ethyl-5-allylthiomethylbarbituric acid	(2) = O, (5)-C_2H_5, (5)-$CH_2SC_3H_5$		3	1	31.5	3.6	8.2	55.3	<0.003
5-Ethyl-5-*iso*amylthiomethylbarbituric acid	(2) = O, (5)-C_2H_5, (5)-$CH_2SC_5H_{11}$		3	1	30.9	3.0	7.8	75.6	<0.003
5-*n*-Propyl-5-*n*-butylthiomethylbarbituric acid	(2) = O, (5)-C_3H_7, (5)-$CH_2SC_4H_9$		3	1	30.6	2.8	8.3	75.4	<0.003
5-*Iso*butyl-5-ethylthiomethylbarbituric acid	(2) = O, (5)-C_4H_9, (5)-$CH_2SC_2H_5$		3	1	31.8	2.9	8.1	68.4	<0.003
5-Methallyl-5-ethylthiomethylbarbituric acid	(2) = O, (5)-$CH_2C(CH_3)=CH_2$, (5)-$CH_2SC_2H_5$		3	1	33.0	3.1	6.9	70.1	<0.003
5-Allyl-5-*α*-ethylthioethylbarbituric acid	(2) = O, (5)-CH_2CHCH_2, (5)-$CH(CH_3)SC_2H_5$		3	2	27.9	3.8	8.8	65.5	<0.003

* 1. Abbott Laboratories 2. American Cyanamid Company 3. Winthrop Chemical Company 4. Parke, Davis & Co.

TABLE 9. AMINOBENZENE DERIVATIVES

Compound	Formula	Source*	No. of tests	Dose mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.	Estimated Activity
4-Aminobenzoic acid	$NH_2C_6H_4COOH$		1	128.	3.8	8.7	31.4	0.003
3,5-Dibromo-4-aminobenzoic acid	$NH_2C_6H_2Br_2COOH$	1	1	24.3	2.2	8.0	91.9	<0.003
β-(4-aminobenzoyl)-propionic acid	$NH_2C_6H_4COCH_2CH_2COOH$	1	1	28.6	3.8	8.7	100.3	<0.003
4-Aminobenzoyl-*l* (+)-glutamic acid	$NH_2C_6H_4CONHCH(COOH)(CH_2)_2COOH$	2	1	158.	1.7	10.1	81.4	<0.003
4,4′-Diaminodiphenylsulfone, didextrose sulfonate derivative (Promin)	$-NH_2C_6H_4SO_2C_6H_4NH_2-$		1	141.	3.2	12.3	23.5	0.007
4,4′-Diaminodiphenylsulfone, disodiumformaldehydesulfoxalate derivative (Diasone)	$-NH_2C_6H_4SO_2C_6H_4NH_2-$	3	1	194.	1.1	8.9	33.8	0.003
4,4′-Diaminodiphenylmethane	$NH_2C_6H_4CH_2C_6H_4NH_2$	1	3	3.9	3.9	10.0	14.7	0.25
Bis-(4-dimethylaminophenyl)methane	$(CH_3)_2NC_6H_4CH_2C_6H_4N(CH_3)_2$	1	7	0.4	3.9	7.8	47.7	0.25
				12.3	3.2	20.2	1.6	
Bis-(4-dimethylaminophenyl)iminomethane	$(CH_3)_2NC_6H_4C(NH)C_6H_4N(CH_3)_2$	1	1	10.1	0.8	6.2	60.2	<0.003
4,4′4″-Hexamethyltriaminotriphenylmethane	$[(CH_3)_2NC_6H_4]_3CH$		1	13.5	3.3	10.5	31.8	0.03
Basic Fuchsin	$(H_2NC_6H_4)_2CC_6H_4NH_2Cl$		1	13.7	3.8	11.0	37.0	0.03
Crystal Violet	$[(CH_3)_2NC_6H_4]_2CC_6H_4N(CH_3)_2Cl$		1	16.5	1.4	8.5	81.6	0.01
Sulfadiazine	$H_2NC_6H_4SO_2NHCNCHCHCHN$	1	1	12.8	2.7	13.8	11.5	0.1
2-Sulfanilamido-5-chloropyrimidine	$H_2NC_6H_4SO_2NHCNCHCClCHN$	1	1	12.2	3.4	12.7	4.5	0.1
2-Sulfanilamido-5-bromopyrimidine	$H_2NC_6H_4SO_2NHCNCHCBrCHN$	1	1	13.7	2.6	11.7	19.8	0.1
2-(3′,5′-Dibromosulfanilamido)-5-chloropyrimidine	$NH_2C_6H_2Br_2SO_2NHCNCHCClCHN$	1	1	27.1	1.9	9.0	100.3	0.003
2-Metanilamidopyrimidine	$H_2NC_6H_4SO_2NHCNCHCHCHN$	1	1	14.9	4.8	9.0	17.1	0.1
2-Amino-5-sulfanilylthiazole (Promizole)	$H_2NC_6H_4SO_2CCHNC(NH_2)S$	2	4	1.2	2.3	9.1	41.3	0.25
				14.5	2.6	18.5	1.8	
2-Acetylamino-5-sulfanilylthiazole	$H_2NC_6H_4SO_2CCHNC(NHCOCH_3)S$	2	1	11.9	2.6	15.0	10.5	0.1

2-Amino-4-methyl-5-sulfanilylthiazole	$H_2NC_6H_4SO_2CC(CH_3)NC(NH_2)S$	2	1	16.1	2.8	11.6	16.9	0.05
2-Methylamino-4-methyl-5-sulfanilylthiazole	$H_2NC_6H_4SO_2CC(CH_3)NC(NHCH_3)S$	2	2	10.9	2.0	10.0	35.7	0.02
2-Sulfanilamido-1,3,4-thiadiazole	$H_2NC_6H_4SO_2NHCNNCHS$	2	1	7.2	2.7	7.2	81.7	<0.01
2-Amino-5-sulfanilyl-1,3,4-thiadiazole	$H_2NC_6H_4SO_7NHCNNC(NH_2)S$	2	2	15.8	3.0	9.3	55.6	0.003
4-Aminomethylbenzenesulfonamide	$H_2NCH_2C_6H_4SO_2NH_2$	4	1	24.2	2.0	7.7	70.6	<0.001
3,5-Dibromo-2-aminobenzoic acid	$NH_2C_6H_2Br_2COOH$	1	1	35.1	2.7	7.9	93.4	<0.001
(Carbamyl-N-thioglycol)-4-aminobenzoic acid	$NH_2C(O)SCH_2C(O)NHC_6H_4COOH$	5	1	22.1	1.0	9.7	86.4	<0.001
(N-thioglycolyl)-4-aminobenzoic acid	$HSCHCONHC_6H_4COOH$	5	1	24.0	2.2	8.1	76.3	<0.001
N-Dithiodiglycolyl-bis-4-aminobenzoic acid	$HOOCC_6H_4NHCOCHS\text{-}SCHCONH\text{-}C_6H_4COOH$	5	1	21.0	1.4	9.1	112.9	<0.001

* 1. American Cyanamid Company. 2. Parke, Davis & Co. 3. Abbott Laboratories. 4. Winthrop Chemical Co. 5. Endo Products

Thiobarbituric acids. Listed in Table 8 are the assays of 39 thiobarbituric acids. These data are sufficiently extensive to establish several structural characteristics as important to the antithyroid effect. In the first place thiobarbituric acid itself is completely inert. This compound might be considered to be 6-hydroxy-2-thiouracil and its lack of activity would be in keeping with that of the other thiouracils which were found to be inactive by virtue of an additional polar group. The inactivity of 5-ethyl-2-thiobarbituric acid may be explained in the same way for this compound also could be considered to be a 6-hydroxy derivative of thiouracil. When both hydrogen atoms at position 5 of 2-thiobarbituric acid are replaced, however, the character of the oxygen atom at position 6 is profoundly modified and it is therefore of interest to note that all of the 5,5-disubstituted 2-thiobarbituric acids tested showed some degree of antithyroid activity. The most active compound in this series was the diethyl derivative; its activity in rats and in man has already been recorded (Astwood, Bissell & Hughes, 1945; Astwood, 1945a). Antithyroid activity decreased sharply as the size of the groups on position 5 were made larger or smaller than the ethyl group.

Eighteen compounds possessing an additional sulfur atom in the form of a thioether side chain were tested. This sulfur grouping did not appreciably modify activity and in this series also the total size of the substituents at position 5 appeared to be the determining factor. Eight barbiturates which lacked a sulfur atom at position 2 were tested and none of these showed any antithyroid activity. The replacement of the oxygen at position 4 by sulfur or by an imino group seemed to decrease activity and the trithio compound was less active than the corresponding 5,5-diethyl-2-thiobarbiturate.

Aminobenzene derivatives. All of the active compounds thus far considered may be classed together; they all contain a thiocarbamyl grouping which seems to be essential for the antithyroid activity. As previously shown there is a second class of antithyroid compounds; this class contains an aminophenyl group and may thus be considered as aniline or aminobenzene derivatives. An examination of Tables 9 and 10 reveals that of the 26 aminobenzene derivatives listed (table 9), 16 were found to possess detectable antithyroid activity. On the other hand of the 16 benzene derivatives lacking an amino group on the aromatic ring (Table 10) none was effective. This is further support for the view that the antithyroid effect is intimately associated with the aromatic amino group.

The slight but consistently detectable activity of 4-aminobenzoic acid was not increased by any of the modifications tried. Likewise no sulfonamide was found to be more active than sulfadiazine which had previously been found to be the most effective sulfonamide.

The most potent compounds encountered in the aminobenzene series were 4,4′-diaminodiphenylmethane, its tetramethyl derivative

TABLE 10. INACTIVE BENZENE DERIVATIVES

Compound	Formula	Source*	Maximal Dose Tested mg./100 Gm. B.W./Day	Daily Body Wt. Gain—Grams	Thyroid Weight mg./100 Gm. B.W.	Thyroid Iodine Concentration mg./100 Gm.
4-Hydroxybenzoic acid	HOC_6H_4COOH	1	128.4	3.3	10.0	54.3
5-Hydroxybenzenesulfonamide	$HOC_6H_4SO_2NH_2$	1	76.8	2.4	9.1	75.6
4-Mercaptobenzenesulfonamide	$HSC_6H_4SO_2NH_2$	1	7.5	2.9	8.2	56.7
β-Hydroxytyrosine	$HOC_6H_4CH(OH)CH(NH_2)COOH$		11.1	3.5	8.5	68.4
4-Methoxyphenylalanine	$CH_3OC_6H_4CH_2CH(NH_2)COOH$		14.1	4.0	7.9	60.1
4-Nitrophenylalanine	$O_2NC_6H_4CH_2CH(NH_2)COOH$		6.8	4.1	7.7	98.0
3,5-Dibromobenzoic acid	$C_6H_3Br_2COOH$	1	30.5	−1.9	9.4	95.4
2,6-Dichloroindophenol	$OC_6H_2Cl_2NC_6H_4OH$		17.1	2.2	6.8	79.9
Benzylhydroquinone	$HOC_6H_4OCH_2C_6H_5$		137.1	1.7	7.9	77.0
β-Hydroxyphenylalanine ethyl ester	$C_6H_5CH(OH)CH(NH_2)COOC_2H_5$		10.4	2.2	7.5	86.3
2-Benzenesulfonamido-4-methylpyrimidine	$C_6H_5SO_2NHCNC(CH_3)CHCHN$	1	6.8	3.0	8.2	71.0
Aminodiphenylacetic acid sodium salt	$H_2NC(C_6H_5)_2COONa$	2	7.1	4.0	8.4	70.8
Ethylphenylsulfone	$C_6H_5SO_2C_2H_5$	2	6.8	3.4	9.1	72.5
Phenethyldodecylsulfone	$C_6H_5CH_2CH_2SO_2C_{12}H_{25}$	2	15.5	3.9	14.6	63.1
2-Benzenesulfonamidothiazole	$C_6H_5SO_2NHCNCHCHS$	1	7.4	3.3	8.1	69.7
Phenylthioacetomorpholide	$C_6H_5CH_2C(S)NCH_2CH_2OCH_2CH_2$	1	4.6	0.8	6.1	70.5

* 1. American Cyanamid Company 2. Parke, Davis & Co.

and promizole or 2-amino-5-sulfanilylthiazole; these were about one fourth as active as thiouracil and thus about twice as active as thiourea.

DISCUSSION

There are several factors which limit the accuracy of the assay method used here for the determination of antithyroid activity. It is well known that the environmental temperature is a major factor determining the intensity of the endocrine function of the thyroid gland and it has been shown (Dempsey and Astwood 1943) that the environmental temperature has a profound influence on the degree of compensatory thyroid hyperplasia following the administration of thiouracil. In the present experiments the environmental temperature was not strictly regulated and this factor doubtless accounted for some of the variations noted and contributed to inaccuracy in the assays. A second factor contributing to variation and to error is the iodine intake. A low-iodine diet greatly augments the effect of an antithyroid compound on the size and iodine content of the gland (Astwood 1945b) and an excess of iodine inhibits slightly the compensatory hyperplasia and decreases greatly the extent of iodine loss from the thyroid gland (McGinty 1945, Astwood 1945b). In the present experiments the iodine intake was not strictly controlled and may have varied because of inconstant concentrations in the commercial diet and in the city water supply. There are other variables such as the relative humidity of the atmosphere, other seasonal factors and the state of health of the colony which doubtless modify the thyroid response but which could not be controlled in these experiments. For these reasons it was of importance that a standard of reference be assayed together with the compounds being tested.

Despite these limitations to the method used it is of interest that assays by this method conducted recently on nearly 120 compounds by McGinty and his collaborators (McGinty and Bywater 1945 a and b and Bywater, McGinty and Jenesel, 1945) agree remarkably well with those reported above. Twenty compounds which were tested in the two laboratories are listed in Table 11. The experiments of McGinty *et al.* were conducted in another city, on a different strain of rats of a younger age. The animals used were obtained commercially and upon arrival in the Detroit laboratory exhibited an abnormally low iodine concentration in the thyroid gland. They were fed a diet which differed markedly from the diet used here and it was supplemented by 1 mg. of potassium iodide per liter of drinking water. The rats were housed in air conditioned quarters at a temperature (25°C.) lower than that which prevailed here. Perhaps because of one or more of these reasons the sensitivity of McGinty's animals was considerably greater than of those used in the experiments done here; the respective dosages of thiouracil expressed in milligrams per 100 gm. of body weight per day required to reduce the concentration

of iodine in the thyroid gland to 12 mg. % were 0.64 and 1.07 indicating that the assays as conducted in Detroit were 1.67 times as sensitive as those carried out in Boston.

In view of this difference in sensitivity it is remarkable to note how well the assays in the two laboratories agree when based upon a comparison with the same standard of reference, thiouracil (Table 11). The values reported by McGinty, *et al.* were calculated on the basis of iodine concentration alone using that portion of the iodine response curve which approximated a straight line.

Eleven of the compounds reported above had been tested previously by the older method (Astwood 1943). While the general order

TABLE 11. THE ASSAY VALUES OF 20 COMPOUNDS TESTED BY MCGINTY ET AL. AS COMPARED WITH THE VALUES REPORTED HEREIN

	Antithyroid activity (thiouracil = 1.00)	
	McGinty et al.	*This report*
5,5-Diethyl-2-thiobarbituric acid	1.23	1.7
2-Mercaptothiazoline	1.31	1.3
5-Amino-2-mercaptothiadiazole	1.56	1.2
6-Methyl-2-thiouracil	1.04	1.
2-Mercaptobenzimidazole	1.16	0.5
1,3-Diethylthiourea	0.47	0.4
2-Amino-5-sulfanilylthiazole	0.18	0.25
Thiourea	0.09	0.12
2-Acetylamino-5-sulfanilylthiazole	0.09	0.1
2-Amino-4-methyl-5-sulfanilylthiazole	0.14	0.05
1-Butylhexahydro-2-thiopyrimidone	0.07	0.01
2-Mercaptobenzothiazole	0.02	0.005
4-Aminobenzoic acid	0.003	0.003
2-Amino-5-sulfanilyl-1,3,4-thiadiazole	0.01	0.003
2,5-Dimercapto-1,3,4-thiadiazole	0.07	<0.03
4,6-Diamino-2-mercapto-*s* triazine	0	<0.01
7-Thio-1,4-diazabicyclo(2,2,1)heptane	0	<0.01
2-Thiobarbituric acid	0	<0.003
3-Phenyl-2-thiothiazolidone	0	<0.003
4-Aminomethylbenzenesulfonamide	0	<0.001

of activity of these compounds was confirmed it appears that the earlier tests under-estimated the activity of thiouracil somewhat. Consequently the previously estimated activities of such compounds as thiourea, acetylthiourea, 4-aminobenzoic acid, sulfadiazine and thioammeline were relatively too high. In the earlier study 38 compounds with detectable activity were found among the 106 substances investigated. In the present study 11 old and 209 new compounds have been tested and among them 115 showed appreciable antithyroid activity. The large number of compounds which proved to possess detectable antithyroid activity among the total of 315 thus far tested here indicates that further and more extensive investigations could profitably be undertaken.

There have been only a few other reports on the antithyroid activity of new compounds. 6-Methylthiouracil has been tested in animals (Chapman, 1944) and man (Leys, 1945) and in agreement with the above findings it proved to be comparable to thiouracil in activity. Higgins and Larsen (1944) first reported the goitrogenic action

of promizole in guinea pigs and rats and recently Williams (1945) has found that tetramethylthiourea and to a lesser degree diethylthiourea are effective in the treatment of hyperthyroidism in man when administered in doses of 0.6 to 1.0 gm. daily. Berman (1945) has reported that the injection of 0.5 to 1.5 gm. of 4-aminobenzoic acid daily is effective in controlling hyperthyroidism. This would imply that this compound is relatively much more active in man than in the rat. 6-Ethylthiouracil and 6-*n*-propylthiouracil have been tested in man on 14 and 37 individuals respectively, and shown to be effective in the treatment of hyperthyroidism (Astwood and VanderLaan 1945). The dosage needed, approximately 75 mg. daily, indicates that these compounds are highly active in man but relatively not quite as active in man as in the rat. Further studies in man are required before any generalization can be made regarding the value of animal assays in the selection of antithyroid agents for use in human beings.

SUMMARY

The relative antithyroid activities of a variety of compounds were determined in rats by administering various doses in the diet for periods of 10 days. The degree of thyroid enlargement and the decrease in thyroid iodine concentration thus induced were used to compare the activities of new compounds with thiouracil which was considered to have an activity of 1.00. Of the 220 substances investigated, 11 had been tested previously, 115 were found to possess detectable activity and 25 proved to be as active or more active than thiouracil. The most potent substances were derivatives of thiouracil; these with their relative activities in decreasing order were:

6-*n* propyl, 11; 6-benzyl, 10; 6-*tert*butyl, 9; 6-*iso*propyl, 9; 6-ethyl, 8; 6-*sec.* butyl, 6; 6-*iso*butyl, 5; 5-ethyl, 3.5; 5-methyl, 6-ethyl, 3.5; 6-*n* butyl, 3; 5-*iso*propyl, 2.5; 5-*n*-propyl, 2; and 5,6-diethyl, 2.

Other compounds appreciably more active than thiouracil were: several further thiouracil derivatives; 5.5-diethyl-2-thiobarbituric acid, 1.7; 2-mercaptoimidazole, 1.5; 2-mercaptothiazoline, 1.3; 5-amino-2-mercapto 1,3,4-thiadiazole, 1.2.

Two types of chemical structure were found to be associated with antithyroid activity. The more active class possessed a thiocarbonamide grouping, the less active an aminobenzene group. Of the latter the most effective substances were: 4.4′-diaminodiphenylmethane, its tetramethyl derivative and 2-amino-5-sulfanilylthiazole. These were approximately one quarter as active as thiouracil.

ACKNOWLEDGMENTS

We are especially indebted to Dr. R. O. Roblin, Jr. and his associates of the American Cyanamid Company, Stamford, Conn. for

supplying the majority of the compounds and for their helpful advice and assistance. Dr. D. A. McGinty and his collaborators of Parke, Davis and Company, Detroit, Mich. kindly allowed us to read their manuscripts before publication and provided a number of compounds for testing. We also wish to thank Drs. D. L. Tabern and E. H. Volwiler of the Abbott Laboratories, North Chicago, Ill., Dr. M. L. Tainter of the Winthrop Chemical Company, Rensselaer, N. Y., Dr. W. A. Lott of E. R. Squibb and Sons, New Brunswick, N. J., Dr. S. M. Gordon of Endo Products, Inc., New York, N. Y., Dr. Treat B. Johnson, Bethwood Research Laboratory, Bethany, Conn., Dr. H. N. Christensen of the Mary Imogene Bassett Hospital, Cooperstown, N. Y., and Dr. C. P. Richter of the Johns Hopkins Hospital, Baltimore, Maryland, for providing compounds for this study.

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NOTES AND COMMENTS

PLASMA PROTEIN CONCENTRATIONS OF RATS FED THIOUREA[1]

A decrease in thyroid activity is invariably followed by a rise in serum or plasma globulin concentration in the rat. This apparent relationship between hypothyroidism and elevated globulin levels has been demonstrated in rats following thyroidectomy, hypophysectomy and also after the feeding of antithyroid drugs (Levin and Leathem, 1942; Levin, 1943; Moore, Levin and Leathem, 1944; Li, 1944; Leathem, 1945; Moore, Levin and Smelser, 1945). A significant rise in plasma globulin is obtained by feeding one per cent thiourea to adult rats for 20 days (Leathem, 1945). However, some suggestion of toxicity was indicated by the marked loss in body weight and the reduction in food consumption. Since these factors could readily influence plasma protein levels, it appeared advisable to re-examine the problem with a smaller dosage of thiourea and compare the results with pair fed as well as with ad libitum fed controls.

Male rats of the Long-Evans strain, ranging from 139 to 162 days in age, were used. These animals were kept in pairs in metabolism cages for the measurement of daily food intake. Thiourea was fed as half of one per cent of the stock diet for a period of 20 to 27 days. The stock diet and its supplements and the manner in which the blood samples were obtained as well as the methods of blood analysis used were the same as those reported previously (Leathem, 1945).

RESULTS AND DISCUSSION

Daily food consumption of the thiourea fed rats was subnormal during at least the first 6 to 8 days of the experimental period and then approached a normal level. Average food consumption per rat during the 20-day period totaled 258 grams for the thiourea fed and consequently for the pair fed normal rats. Normal rats eating ad libitum consumed an average 340 grams of food during the same period. The thiourea rats lost weight and at the end of 20 days the body weight loss averaged 46 grams. Pair fed controls however, lost, on the average, only 19 grams in weight and thus factors other than reduced food intake are stimulating a body weight loss when thiourea is fed. The control rats eating ad libitum uniformly gained weight (Table 1).

Thiourea caused an increase in total plasma protein due to an increase in plasma globulin concentration in comparison with pair fed and ad libitum fed controls (Table 1). Plasma albumin was subnormal but the lowered

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[1] Publication of the Bureau of Biological Research, Rutgers University.

hematocrit suggests a hemodilution which could account for the decrease in plasma albumin and if correction was permitted would even further increase the plasma globulin concentration. Blood protein studies on the pair fed rats revealed the typical effects of reduced food intake. Total plasma protein was reduced due entirely to a decrease in plasma albumin concentration. Thus the rise in total plasma protein due to a rise in plasma globulin is not due to reduced food intake. Non-protein nitrogen is above normal in thiourea fed rats.

The rise in plasma globulin concentration as related to hypothyroidism is further substantiated by Lewis and McCullagh (1944). These investigators studied the plasma proteins of hypothyroid patients by electrophoretic analysis and found also that adequate thyroid therapy would return the blood proteins to normal levels. Electrophoretic fractionation of rat serum

TABLE 1. INFLUENCE OF THIOUREA ON PLASMA PROTEIN LEVELS OF RATS

No. of rats	Treatment	Body weight start–end	Hematocrit	Non-protein nitrogen	Total Protein	Albumin	Globulin	A/G Ratio
		gm.	per cent	mg./100 cc.	gm./100 cc.	gm./100 cc.	gm./100 cc.	
20	thiourea	303–257	41.6±0.7[1]	68.0±2.2	6.57±0.08	3.56±0.08	3.02±0.08	1.14±0.05
20	none pair fed	301–282	45.7±0.5	60.0±2.0	6.17±0.06	3.47±0.05	2.69±0.07	1.29±0.05
20	none—fed ad. lib.	255–272	45.4±0.7	53.5±0.8	6.37±0.07	3.86±0.09	2.51±0.10	1.54±0.10

[1] Mean deviation of mean $= \epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

reveals that the α globulin component is present after removal of the thyroid or hypophysis but is rarely present in normal rat serum (Moore, Levin and Leathem, 1944; Moore, Levin and Smelser, 1945).

It is well known that inanition may alter the blood protein concentrations generally by decreasing the albumin level (Weech, 1938). The reduction of food intake to which the pair fed normal rats were subjected was sufficient to lower the plasma albumin concentration. Lewis and McCullagh (1944) noted a low albumin in their hypothyroid patients. Recently, Moore, Levin and Smelser (1945) reported a significant rise in serum albumin after thiouracil feeding but this has not been observed by us with thiourea.

SUMMARY

A rise in plasma globulin concentration occurs in male rats fed a half of one per cent thiourea for 20 to 27 days. A rise in total plasma protein and non-protein nitrogen is also produced. Thiourea causes a reduction in food intake but pair fed rats reveal that the rise in plasma globulin is not due to reduced food intake. Total plasma protein of pair fed rats is less than that of ad libitum fed rats due to a decrease in plasma albumin concentration.

Thiourea feeding reduces body weight beyond that which can be accounted for by the decreased food consumption.

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ABSENCE OF PARAXANTHINE EFFECT ON METABOLIC RATE OF RATS

Since the early observations that sulfanilamide and thiourea derivatives inhibited thyroid functioning, much interest has centered upon increasing the variety of drugs producing this effect. Such compounds as thiocyanate, sulfanilamide, para-amino benzoic acid and thiourea have been found effective. Carter, Mann, Harley-Mason and Jenkins (1943) reported that 1,7-dimethyl xanthine (paraxanthine) exerted a marked anti-thyroxin action in rats in such extremely small doses as 100μg. per kg. per day. This work not only implicated a type of compound commonly occurring in the mammalian organism but also suggested that the peripheral action of thyroid hormone could be inhibited.

Williams (1945) has recently reported his inability to detect any antithyroid activity of paraxanthine on the metamorphosis of tadpoles, on hyperthyroid patients, or on the respiration of liver tissue exposed in vitro to thyroglobulin. We have repeated the work of Carter et al. with rats as closely as possible, and have also been completely unable to confirm their results.

Methods. Young adult albino rats weighing 200–300 gm. and bred from the Sprague-Dawley strain were used. These animals were carefully trained for use in the Benedict multiple-chamber metabolism apparatus. In addition, they were accustomed to consuming their entire day's food in 5 hours so that metabolism determinations could be made on several consecutive days. Paraxanthine[1] was administered as specifically mentioned later.

Experimental. 1. Since Carter et al. stated that a dose of about 100 μg. paraxanthine per kg. body weight, given by mouth, would decrease the metabolic rate of the rat by 25–30%, the first series of experiments consisted of the oral administration, in 3 divided doses, of this amount of the material to 8 male and 8 female rats. As Table 1 shows, no effect was obtained in 3 weeks of this procedure. Variations were then introduced, as noted in Table 1, consisting of (a) subcutaneous injection of the compound, (b) reduction of the dose to one-half of the original amount, and (c) increase of the dose to double. Still, no consistent alterations in BMR were produced. In a few animals (not shown in the table) the paraxanthine was adminis-

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[1] Paraxanthine was synthesized from caffeine by R. C. Combs of the Department of Chemistry. It had a melting point of 286°C. itself and 287°C. when mixed with an equal amount of the material synthesized by the American Cyanamid Company (Williams, 1945).

tered in the drinking water, without any deviation from the negative results.

As can also be seen from Table 1, two other groups, of 4 males each, were given ten times and one hundred times the original dose, respectively, without change in oxygen consumption.

The thyroids of representative animals from each of these groups were

TABLE 1. OXYGEN CONSUMPTION OF RATS ON PARAXANTHINE

Condition of animals	Paraxanthine μg./kg./day	Oxygen consumption[1] Males	Females
Group A—8 males, 8 females			
Aver. of 3 prelim.	0	100.1	104.4
13 d. on paraxan. orally	100	99.7	105.5
21 d. on paraxan. orally	100	97.9	104.2
7 d additional subcu.	100	99.3	100.8
7 d. additional orally	50	98.3	103.0
7 d. additional orally	200	97.8	102.4
Group B—4 males			
Aver. of 2 prelim.	0	103.7	
8 d. on paraxan. orally	1,000	99.4	
18 d. on paraxan. orally	1,000	102.1	
Group C—4 males			
Aver. of 3 prelim.	0	100.4	
8 d. on paraxan. orally	10,000	96.9	
18 d. on paraxan. orally	10,000	96.5	

[1] ml. of oxygen per 100 gm. body wt. per hour.

TABLE 2. EFFECT OF PARAXANTHINE ON EXPERIMENTAL HYPERTHYROIDISM

Condition of animals	Paraxanthine μg./kg./day	Oxygen consumption ml./100 gm./hour
Aver. of 2 prelim.	0	96.8
5 d. on desiccated thyroid	0	174.3 (+80.2%)
8 d. on desiccated thyroid	500, 3 days	179.1 (+85.0%)
11 d. on desiccated thyroid	250, 3 days	173.8 (+79.6%)
14 d. on desiccated thyroid	1,000, 3 days	177.3 (+83.3%)

found to exhibit a normal histological picture, in contradistinction to the definite alterations produced by thiourea and thiouracil.

2. An attempt was next made to duplicate the original report (Carter et al., 1943) that experimental hyperthyroidism could be reduced by paraxanthine, a doubled BMR being returned to normal by 100 μg. per day (presumably, 500 μg. per kg. per day). After their resting metabolic rates had been determined, 8 male rats were given desiccated thyroid[2] in amounts raising the BMR about 80%. This was found to be about the peak metabolic rate which could be maintained without greatly increasing the animals' activity. As shown in Table 2, the subsequent administration of 250, 500 or 1000 μg. of paraxanthine (again divided doses) per kg. per day produced no change in the accelerated metabolism.

[2] Furnished by Dr. I. J. Klingaman of the Cudahy Packing Company.

As further check, 12 rats previously receiving paraxanthine in amounts from 50 to 10,000 μg. per kg. per day were given by stomach tube a single dose of 1000 mg. of desiccated thyroid per kg. body weight and the metabolic rate studied 2, 3 and 4 days later. Eighteen control animals gave an average increase of 36.6%. The 12 animals on paraxanthine showed an average response of 35.9%. Thus, paraxanthine in this wide range of doses did not appear to interfere with the effects of administered thyroid substance.

3. In a later report (Carter and Jenkins, 1944) the English workers reiterated their belief in the existence of a natural anti-thyroid material, but implied some doubts as to paraxanthine itself being the compound. Since a sample of 1,7-dimethyl guanine (as the hydrochloride) was available,[3] this material was tried with a smaller number of animals in nearly all of the combinations used with paraxanthine. It exhibited no ability to decrease the BMR or to interfere with the elevation of metabolism produced by desiccated thyroid. No table of data is included, inasmuch as the figures so closely resemble those with paraxanthine.

Summary. 1,7-dimethyl xanthine (paraxanthine) was found to exert no depressant effect on the BMR of albino rats when given in amounts of 50 to 10,000 μg. per kg. body weight per day. Furthermore, paraxanthine in the same range of doses did not alter the usual increase in oxygen consumption following the administration of desiccated thyroid substance.

1,7-dimethyl guanine also showed a lack of anti-thyroid activity.

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A STUDY OF THE TOXIC, GOITROGENIC AND GROWTH-RETARDING EFFECTS OF THREE DERIVATIVES OF THIOURACIL IN THE ALBINO RAT

Three drugs, chemically related to thiouracil (2-thio, 4-oxy pyrimidine), have been fed in single, daily doses to albino rats to determine their goitrogenicity and possible toxic effects. These drugs were 2-thio, 4-oxy, 6-methyl-pyrimidine, 2-thio, 4-oxy, 5, 6-dimethyl-pyrimidine and 2-thio, 4,5-dimethyl, 6-methyl-ethyl-pyrimidine. These drugs are hereinafter referred to as drugs I, II-and III, respectively.

The goitrogenic index was determined as the ratio of thyroid weight/body weight of treated animals to thyroid weight/body weight of normal animals. (A figure of 1.2 or above was believed to be significant of goitrogenic activity.) The normal thyroid weight/body weight ratio was based upon an average of determinations made in 23 rats.

[3] Through courtesy of Dr. B. K. Harned of the Lederle Laboratories.

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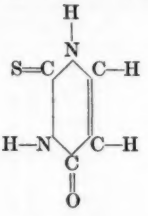
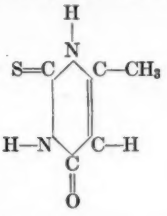
Thirteen rats received drug I over periods of 23 to 28 days in doses varying from 0.05 to 0.2 gms. daily. In all, a significant increase in the thyroid weight was observed. The averaged goitrogenic index for the group was 1.6. The goitrogenic index for rats fed thiouracil in identical dosage over similar periods of time was 1.6. The average gain in body weight of the prepubertal animals was 30% of that noted for their prepubertal controls.

A definite tendency to precipitation of drug I in the kidney with damage to the tubular epithelium was noted in a high percentage of the subjects on doses of 0.1 gm. or more daily. Thiouracil in similar amounts, showed no demonstrable toxic effect.

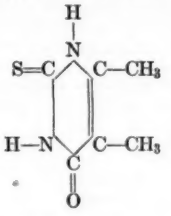
An increase in thyroid weight was observed in each of ten rats receving drug II over periods varying from 23 to 28 days with daily doses of from



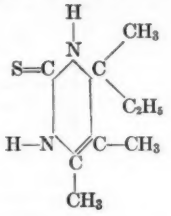
Thiouracil or 2-thio, 4-oxypyrimidine



6-methyl thiouracil or 2-thio, 4-oxy, 6 methyl pyrimidine



5,6-dimethyl thiouracil or 2-thio, 4-oxy, 5,6-dimethyl pyrimidine.



2-thio, 4,5-dimethyl, 6-methyl ethyl pyrimidine

0.05 to 0.2 gms. The average goitrogenic index was 1.2 compared with an average goitrogenic index of 1.7 obtained for animals fed corresponding doses of thiouracil over similar periods of time. This drug did not exhibit any growth-retarding effect. Post-mortem studies revealed some damage to the tubular epithelium of the kidney and significant vacuolization of cells in the center of the hepatic lobules.

On microscopic examination the thyroid glands of the rats treated with drugs I and II showed varying degrees of hyperplasia and diminution of colloid.

Five rats were given drug III in doses of 0.15 to 0.2 gms. daily. All of the rats died within a 4 day period. At autopsy large collections of serous fluid were noted in all the body cavities. Because of the obviously high toxicity of the drug and its probable low therapeutic index, further experimentation with it was abandoned.

When the data obtained by studying these drugs was compared with the known action of thiouracil their relatively greater toxicity was readily apparent. No one of the three can be offered as a satisfactory substitute for the treatment of human beings with toxic goiter.

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ALIMENTARY GLYCOSURIA IN THE RAT

This report represents a preliminary study of the ability of the rat to tolerate a force-fed high carbohydrate deit and of the amounts of glucose which were excreted in the urine when the assimilation limit was exceeded.

METHODS

Six male rats of the Sprague-Dawley strain were maintained on Purina Dog Chow until they reached a weight of 300 grams. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:00 A.M.) and late afternoon (4:30 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1). The diet was made according to Table 1.

TABLE 1. CONSTITUENTS OF THE FLUID DIET USED IN FORCE-FEEDING

Constituent	Grams
Cellu Flour (Chicago Dietetic Supply)	120
Osborne and Mendel salt mixture	40
Wheat germ oil	10
Mazola Oil	10
Cod liver oil	10
Dried yeast (Pabst)	100
Egg albumin (Merck)	160
Starch	500
Dextrin	250
Sucrose	250
Water to make total volume of	2,000 cc.

The animals were brought to the normal feeding of 26 cc. per day on the fifth day. This level of food intake was continued for seven days and thereafter the amount was increased by 1 cc. per day per rat until the death of the animal.

Urinary glucose was determined by the method of Benedict (2).

RESULTS

The data are summarized in Figure 1. Rats 101 and 602 died immediately following the development of glycosuria. At necropsy these animals were found to have extensive areas of hemorrhage involving the thymus, walls of stomach and the pancreas. The remaining four rats sustained glycosuria for periods up to fifteen days prior to death. Values for urinary glucose up to 6.5 grams per day were observed. With the exception of rat 101, each animal exhibited a sharp loss in weight due to dehydration prior to death. All of the rats were free from diarrhea throughout the experiment.

DISCUSSION

Normal rats can assimilate very large amounts of orally administered carbohydrate when time is permitted for adaptation. Rat 201 showed the smallest assimilation limit, yet, at a body weight of 400 grams, it tolerated up to 41 cc. of diet per day prior to the development of glycosuria. If it is assumed that all of the glucose derived from the carbohydrate, protein and glycerol of the diet is transported by the blood during assimilation it can

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be calculated that rat 201 removed from its blood approximately 24 grams of glucose per twenty-four hours or an average of 250 mgm. per 100 grams of rat per hour. In the other rats these figures are greater. It is probable that the rate of assimilation was highest during the early hours following feeding. These data likewise show that after the assimilation limit has been exceeded considerable amounts of glucose can be excreted in the urine. The cause of

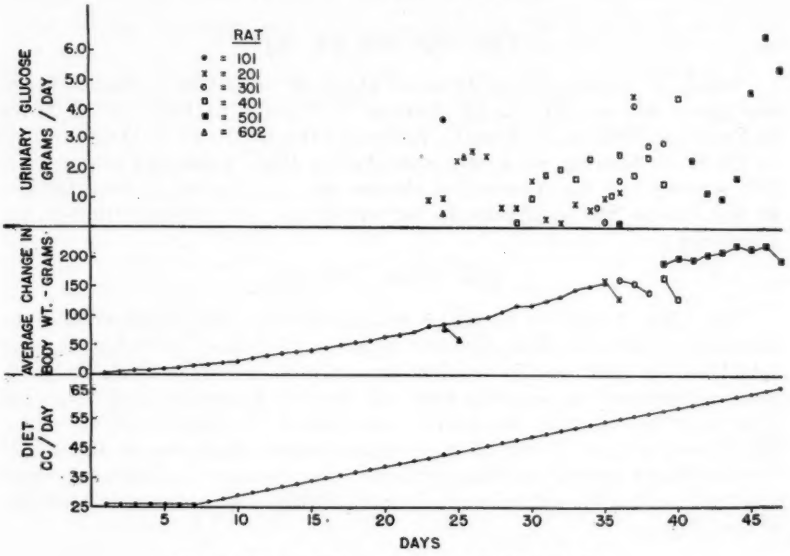

FIG. 1. Urinary Glucose in Force-fed Intact Rats.

the diffuse hemorrhages observed in rats 101 and 602 is not known although trauma can be excluded as a cause.

SUMMARY

Six normal male rats were force-fed a high carbohydrate diet in gradually increasing amounts until death. Very large amounts of diet were tolerated by each rat but after the assimilation limit was past each animal excreted significant amounts of glucose in the urine.

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ASSOCIATION NOTICE

THE SQUIBB AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, in 1942 to Dr. Fred C. Koch, in 1944, to Dr. F. A. Doisy, in 1945 to Dr. E. C. Kendall, no Award was given in 1943. A special Committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. Dr. E. B. Astwood received the award in 1944, Dr. Jane A. Russell in 1945. The work cited may be either in the field of pre-clinical or clinical endocrinology. The Award is for $1,200.00. If the recipient should choose to use the Award toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00. The option is left entirely to the recipient.

Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications, and reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, not later than March 15, 1946.

CARL R. MOORE, *President*
HENRY H. TURNER, *Secretary*

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